Third party approach using cord blood Tregs in allo-transplantation

A thesis submitted in fulfillment of the requirements for

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I, Daniel Figueroa Tentori, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

DEDICATION

This thesis is dedicated to my lovely wife "Annie" and my son "Santiago". Annie, there are countless reasons I want to personally thank you for. Herein, I want to emphasize my gratitude on your undoubted and dauntless decision of joining me in my training journey away from home, with all its inherent hurdles that implies doing such. Your love and endless support has been cornerstone for the achievement of this work.

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Abstract

Compelling data has demonstrated the fundamental role of regulatory T cells (Tregs) in immuno-regulation. In vivo animal models indicate that recipient's $CD4^{pos}$ $CD25^{pos}$ T cells adoptively transferred can prevent or control graft versus host disease (GvHD) and allograft rejection. Furthermore, it has been demonstrated that $CD4^{pos}$ $CD25^{pos}$ T cells from a third party could also be used as an adoptive cell therapy to ameliorate allo-responses. Compelling data supports that $CD45RA^{pos}$ Tregs represent the most homogenous population among the overall Treg pool.

The objective of this study is to test the "Third party approach" using CB Tregs to suppress alloresponses. Herein is shown that CB Tregs were mainly $CD45RA^{pos}$ $CD31^{pos}$ (>80%), which specifically depicts RTE cells that confer a wide TCR repertoire. This study shows an optimized one-step isolation method using anti-CD25 microbeads that achieves high purity (90%) for $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ T cells and decreases substantially the level of effector T cells (<9% of $CD4^{pos}$ $CD127^{high}$). Due to the low frequency of Tregs (1% from overall lymphocytes), the modality of pooling mismatch CB units for Tregs isolation was tested. The pooled CB Tregs showed constitutively potent suppression ability *in vitro*. Interestingly, in 40% of the cases a better suppression was seen with pCB Tregs compared to individual CB Tregs suggesting a "synergetic effect". In summary, this study suggests that CB units fulfill the optimal properties for the isolation of *bona fide* Tregs, which can be isolated with the highest purity using a single step isolation method under GMP standards. Moreover, this study suggests that CB Tregs can be intentionally *pooled* and tailored under the required HLA matches for clinical settings.

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The following abbreviations were used throughout this thesis. Most common general abbreviations of units or names used are not listed here.

ANRI	Anthony Nolan Research Institute
Aka	also known as
APC	antigen presenting cells
BSA	bovine serum albumin
BD	Becton Dickinson
CB	cord blood
CBMCs	cord blood mononuclear cells
CPD	citrate-phosphate-dextrose
CFSE	carboxyfluorescein diacetate succinimidyl ester
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated Ag-4
DCB	double cord blood
DC	dendritic cells
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
FoxP3	forkhead box P3 transcription factor
FSC	forward side scatters
GvHD	Graft versus Host Disease
GITR	glucocorticoid-induced tumor receptor
GMP	Good Manufacturer Practice
HSCT	Hematopoietic Stem Cell Transplantation
hiFCS	heated inactivated fetal calf serum
HLA	Human leukocite antigen
IMD	inherited metabolic disorders
ICOS	inducible T cell costimulator
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
LAP	latency associated peptide
MHC	Major Histocompatibility Complex

miH	minor histocompatibility antigens
MFI	mean fluorescence intensity
NIMA	non-inherited maternal antigens
PFA	paraformaldeheyde
PBMCs	peripheral blood mononuclear cells
pCB	pooled cord blood
PD-1	programmed cell death-1
PAMPs	pathogen-associated molecular patterns
PMA/Io	phorbol 12-myristate 13-acetate/ Ionomycin
RA	rheumatoid arthritis
ROR)γt	retinoid-related orphan receptor γ transcription factor
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
RTE	recent thymic emmigrant
SSC	size scatter
SOT	Solid Organ Transplantation
Th	T helper
T-bet	T box transcription factor
Tregs	regulatory T cells
TM	transport media
TCR	T cell receptor

CHAPTER 1

INTRODUCTION

The grounding studies by Peter Gorer and George Snell discovered the involvement of the major histocompatibility complex (MHC) molecules in the rejection of transplanted tumors (Gorer, 1948; Snell *et al.*, 1951). Subsequently, it was shown by R. Zinkernagel and P. Doherty (Zinkernagel *et al.*, 1975), that the nature of T cell-mediated immunity towards virus-infected cells was necessarily through the recognition of self-MHC, process called "*self MHC restriction"*. However, the adoptive transfer of cells or tissue between two genetically diverse individuals also evokes an adaptive and specific immune response towards non-self MHC/peptide complexes. As many as 10% of donor or host T lymphocytes, main participants in allo-response, can strongly recognize MHC molecules expressed on the transplanted cells or within the host (Lechler *et al.*, 2005). Such recognition can evoke a by-directional immune response; 1) Host vs Graft and 2) Graft vs Host (Starzl *et al.*, 2001). These immune responses will be dependent of the graft transplanted and the host immune-competency.

1.1 Major Histocompatibility Complex

The primary function of MHC molecules is to elicit an immune response by presenting antigenic peptides for recognition by T cell receptors (TCR). Each MHC molecule consists of an extracellular peptide-binding cleft, followed by an immunoglobulin-like domains and transmembrane and cytoplasmic domains. MHC genes are the most polymorphic genes present in the genome and

contains > 200 genes, which are located on the short arm of chromosome 6 at 6p21.3 and mostly are related to immunity (Klein *et al.*, 2000). In humans, these genes are called ***h***uman ***l***eukocyte ***a***ntigen (**HLA**). There are divided in: HLA class I (telomeric end), HLA class II (centromeric en d) and HLA class III (lying between class I and II). Class I contains the genes for HLA-A, -B, and –C and class II contains HLA-DR, -DP and –DQ (Figure 1). MHC molecules class I present peptides endogenously synthesized, wherein MHC molecules class II present extracellular antigens endocytosed into vesicles (Engelhard, 1994). Therefore, MHC class I molecules present peptides to and are recognized by $CD8^{pos}$ T cells, and MHC class II molecules present peptides to $CD4^{pos}$ T cells.

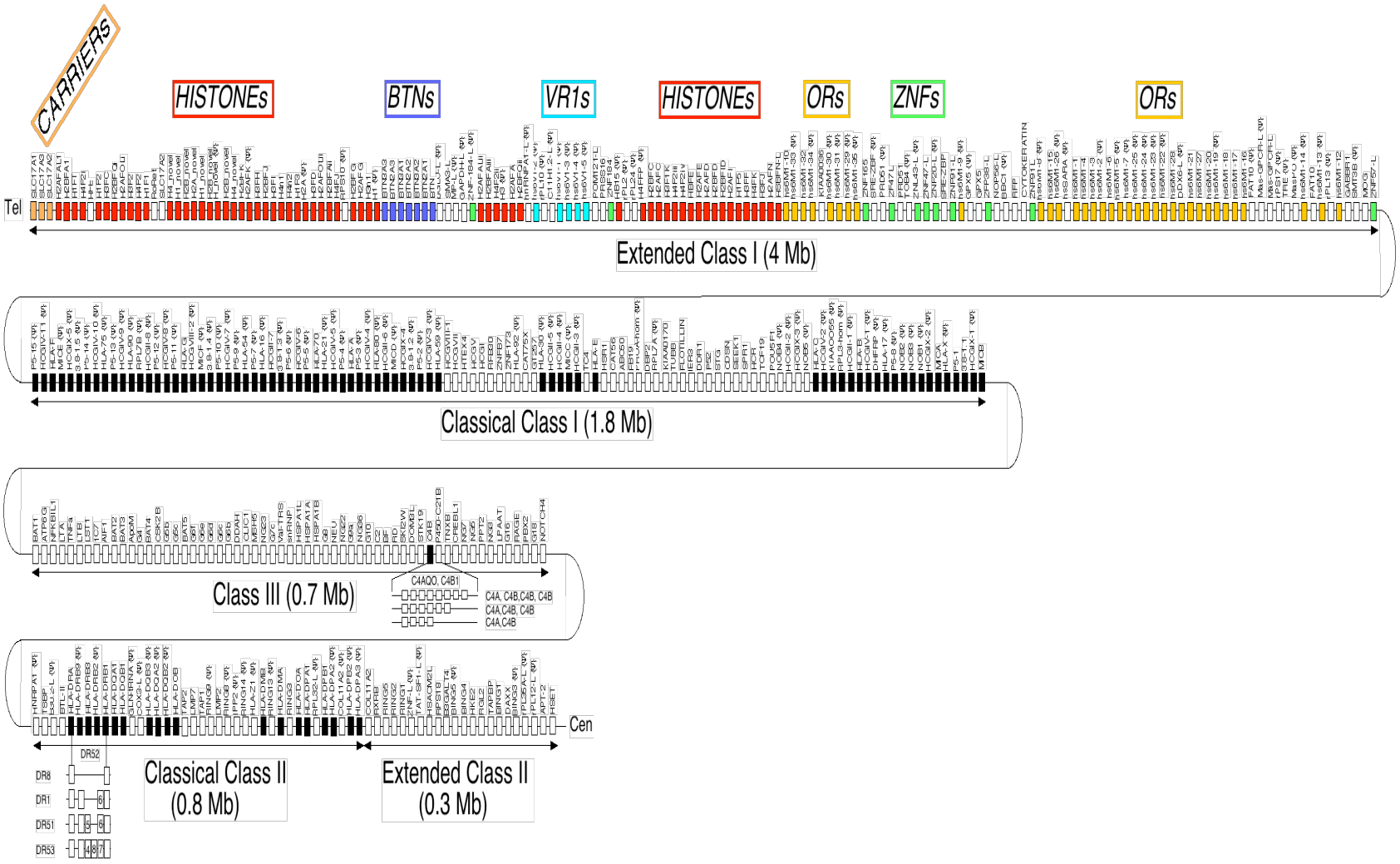


Figure 1.1 MHC gene structure. Figure adapted from John Trowsdale (personal communication).

1.2 Innate and adaptive immune system

The innate immune system provides the first critical mechanisms that allows, a rapid sensing and discrimination between self and non-self (Akira *et al.*, 2006; Janeway *et al.*, 2002; Medzhitov *et al.*, 2002). The main effector cells of the innate immune system are the neutrophils, mononuclear phagocytes and natural killer (NK) cells. The pathogenic substances that stimulate innate immunity are called pathogen-associated molecular patterns (PAMPs), which are captured by pattern recognition receptors. The cellular effectors from innate immunity, mainly macrophages and NK cells, secrete a wide variety of cytokines (ie., IFN-γ, TNF, IL-1, IL-12, etc) that elicit an inflammatory cascade (Janeway *et al.*, 2002). However, this system is frequently overwhelmed due to the diverse antigenic exposure to which the human being is faced with throughout life. This has driven the evolution of the adaptive immune system, which is mediated by B cells and T cells. The capture of antigens by antigen presenting cells (APC) and subsequently their transportation to secondary lymphoid organs, are the first steps in adaptive immunity (Inaba *et al.*, 1984; Steinman, 1991). In contrast to innate immunity, adaptive immunity effectors, primarily T cells, confers a wider repertoire for antigen recognition (Janeway *et al.*, 2002)(10^3 molecular patterns and 10^7 distinct antigens, respectively). The major subsets of B cells are marginal zone B cells, follicular B cells and B-1 B cells, each subset is localized in different location within the lymphoid tissues (Hardy *et al.*, 2000). Likewise, there are three main T cell subsets described as helper T lymphocytes (Th), cytotoxic T lymphocytes (CTLs) and $CD4^{pos}$ regulatory T cells (Tregs) (Mosmann *et al.*, 1989; Sakaguchi *et al.*, 2008). B and T cells have clonally distributed antigen receptors that have been created from recombination of DNA

segments during their maturation (Goldrath *et al.*, 1999; Jung *et al.*, 2006). For the purpose of this study I will only focus on the contribution of T cells in the adaptive immune system.

1.2.1 T cell receptor

TCRs are highly variable antigen-recognition structures. Most mature T cell present a disulfide-linked membrane-bound heterodimers consisting of α and β chains (Call *et al.*, 2005). Each chain is the result of rearrangement of multiple copies of variable (V), joining (J) and in the case of the β-chain, diversity (D) segments during T cell maturation and subsequent joining to one of the constant (C) regions (Goldrath *et al.*, 1999). The α and β chains within the TCR of a mature T cells, contains an amino-terminal variable (V) region and a constant (C) region, followed by a short hinge region, a transmembrane region and a cytoplasmic tail (Figure 1.2).

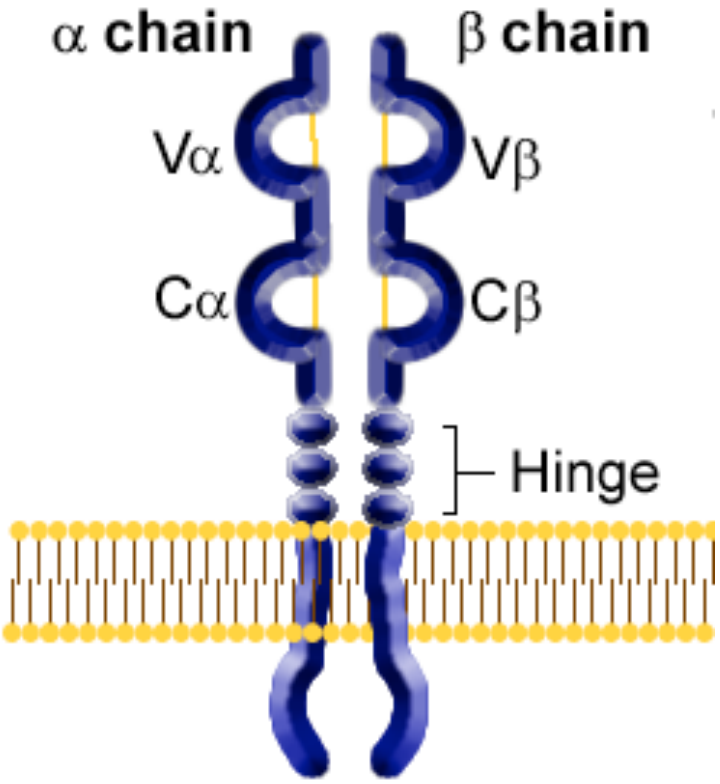


Figure 1.2 Structure of T cell receptor. This illustration was adapted and can be found on bioweb.wku.edu/courses/ biol328/TcR.htm.

1.2.2 T cell activation

T cells are activated through the interaction of their TCR with the antigenic peptides within the MHC molecule, process determined as Signal 1. $CD8^{pos}$ T cells interact with peptides on almost any cell expressing MHC class I. These MHC class I-restricted peptides are produced from proteins translated within the cell encoded either by the host or by infecting pathogens that are replicating inside the cell. Conversely, $CD4^{pos}$ T cells recognize peptides bearing MHC class II that derive from extracellular proteins that were internalized into the vesicles of APCs. MHC class II molecules are present on APCs and are inducible by the innate immune stimuli. In addition to TCR/MHC peptide complex

interaction, T cell activation also requires costimulatory signals, mainly via interactions between CD28 on T cells and CD80/CD86 molecules expressed on APCs (Signal 2) (Sharpe, 2009). CD28-mediated signals, enhance T cell autocrine secretion of growth factor IL-2 and differentiation of naïve T cells into effector and memory T cells. In addition, CD28 favors T cell survival via the expression of the antiaoptotic protein Bcl-x. However, there have also been described certain homologues molecules that in contrast to CD28 confer inhibitory signals (CTLA-4 and PD-1 described later on).

More recently, it has been shown that the presence of an inflammatory cytokine milieu (ie., IL-1, IL-6, IFNα/β, IL-12, IL-21) also plays a major role in T cell activation and differentiation (Signal 3) (Ben-Sasson *et al.*, 2009; Curtsinger *et al.*, 2010).

1.2.3 $CD4^{pos}$ T cell lineages

$CD4^{pos}$ naïve T cells upon encounter with their cognate antigen have the ability to respond in different ways, and to do so they differentiate into a variety of effector subsets (Zhou *et al.*, 2009a) (Figure 1.3). This process of differentiation is influenced greatly by the cytokines in the microenvironment and the strength of TCR interaction with the antigen upon. Th1 cells are characterized by their production of IFN-γ and are involved in cellular immunity against intracellular pathogens. The secretion of IL-12, produced by the innate immune cells as well as IFN-γ produced by NK and T cells, polarize cells towards a Th1 response through the action of the signal transducer and activator of transcription 4 (Stat4), Stat1, and T box transcription factor T-bet. In contrast, Th2 cells are

required for humoral immunity to control extracellular pathogens and their main features are the secretion of IL-4, IL-5 and IL-13. Th2 differentiation requires the action of GATA3 downstream of IL-4 and Stat6. Recently it has been described a Th17 subset (Harrington *et al.*, 2005), which main characteristic is the secretion of IL-17A, IL17F and IL-22. These cells play an important role in clearance of extracellular bacteria and fungi, specifically at mucosal surfaces. Th17 differentiation requires retinoid-related orphan receptor (ROR)γt (Ivanov *et al.*, 2006), a transcription factor that is induced by TGF-β in combination with IL-6, IL-21 and IL-23 all of which activate Stat3 phosphorylation. Follicular helper T (Tfh) cells are a subset that regulates the maturation of B cell responses. Tfh differentiation requires the cytokine IL-21 and it has been associated with the transcription factor Bcl-6 (Yu *et al.*, 2009b). Lastly, the initial effector T cell responses, is often followed by a counter regulatory subset to limit potential collateral damage to the tissues. These cells are called induced Tregs (iTregs) and will be discussed later on.

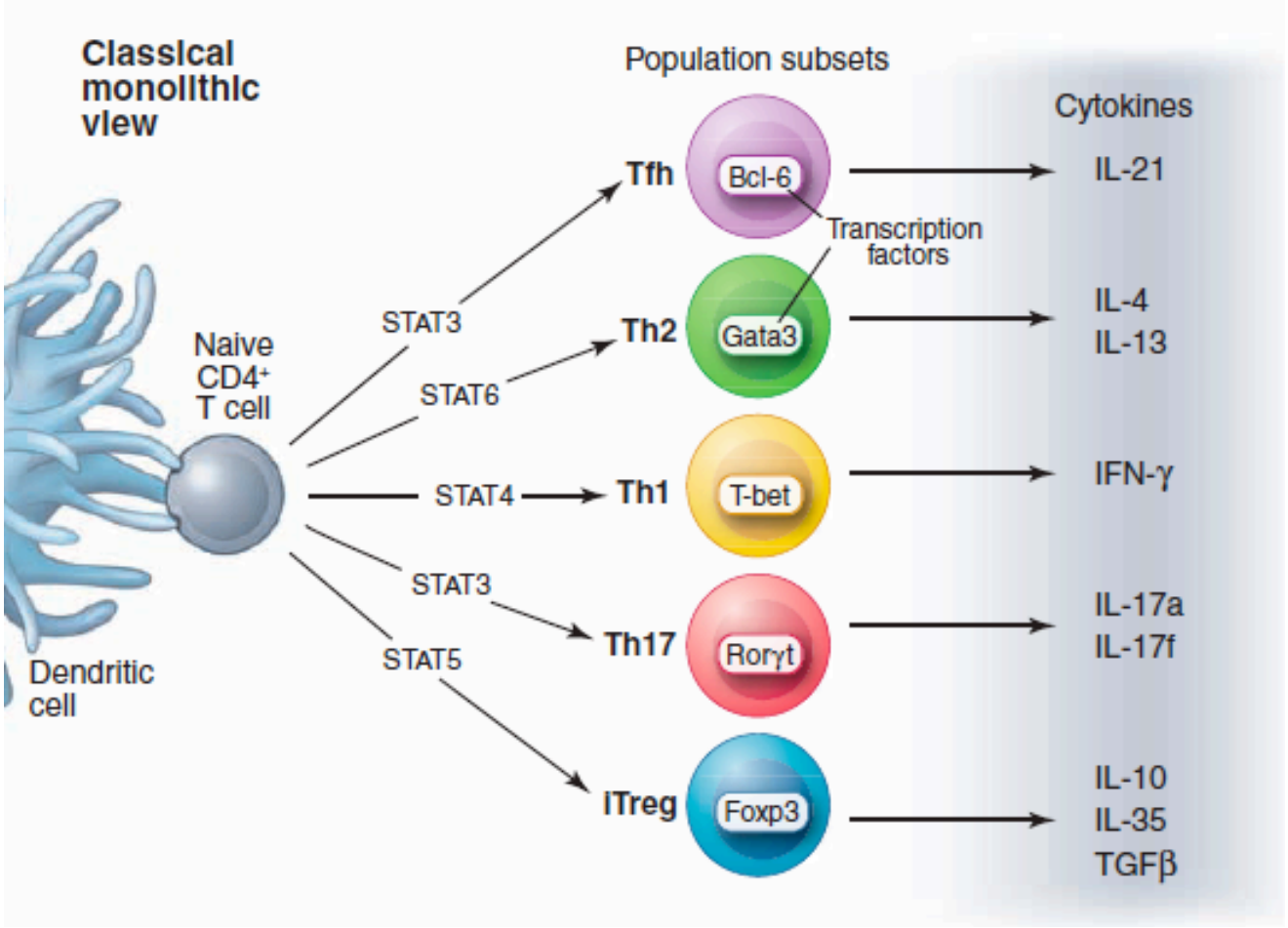


Figure 1.3 CD4^{pos} T cell lineages. This figure illustrates the differentiation pathway of helper T cells. The according transcription factors and cytokines from each subset are exemplified. Adapted from (O'Shea *et al.*, 2010).

1.2.4 $CD4^{pos}$ T cell plasticity

As shown previously, it was common knowledge that T cell differentiation is irreversible and governed predominantly by the cytokines within the microenvironment and by the TCR strength interaction with their cognate antigen (Boyton *et al*., 2002). It is clear that Th1 cell cytokines supress Th2 cell differentiation through a feed-forward mechanism. IFN-γ induces T-bet, which in turn induces Runx3 expression. Runx3 in addition of T-bet, promotes IFN-γ production while silencing the Il4 gene in Th1 cells by binding to the IFN-γ promoter and the Il4 silencer, respectively (Djuretic *et al*., 2007). However, current knowledge regarding two new lineages, induced Tregs and Th-17 T cells, has shown conflicting levels of plasticity. In a murine model was shown that a scarce population, $CD25^{neg}$ $FoxP3^{pos}$ T cells, downregulated FoxP3 once adoptively transfered in vivo and was able to produce detectable levels of IL-17 and IFN-γ (Komatsu *et al*., 2008). Likewise, it has been shown in human naive Tregs $CCR6^{pos}$ population, the production of IL-17 once activated in the presence of the pro-inflammatory cytokines IL-1β and IL-6 (Berious *et al*., 2009). Noteworthy, the aforementioned plasticity between Tregs and Th-17 T cells, comes from studies performed at the population level. Moreover, since there is no specific marker for Tregs in able to achieve a 100% purity, a concise differenciation between plasticity or outgrowth of contaminant Th cells cannot be attained.

1.3 Allorecognition

Allorecognition refers to the phenomenon by which the immune system from the host reacts to non-self MHCp complexes from the donor (Afzali *et al.*, 2007). This phenomenon can be elicited through three main mechanisms (Figure 1.4).

1.3.1 Direct-pathway

It refers to the process when host T cells recognize intact donor MHC molecules presented at the surface of donor cells, mainly APCs that were transplanted within the graft. The structural basis of T cell direct-allorecognition has led to two main theories (Afzali *et al.*, 2008; Archbold *et al.*, 2008). The first one called “antigen density model “ proposed by Bevan. This model features the allo-MHC molecule as the cornerstone for allorecognition and suggests that engrafted cells contain high concentration of allo-MHC molecules, whereto an alloreactive T cell will recognize the inherent disparities within the MHC molecule as antigenic creating an immune response against the graft, all this independently of the bound peptide. This theory is supported by studies where blocking TCR contacting regions from allo-MHC inhibits alloresponses. In addition, alloreactivity can be elicited in the absence of a bound peptide to the all-MHC molecule (Smith *et al.*, 1997). This peptide independent theory would explain the higher frequency of alloreactive T cells in contrast to the lower frequency of most antigen-specific T cells that focus their recognition upon peptide. The second theory called “multiple binary complexes”, suggests that allorecognition is mostly peptide-dependent (Matzinger *et al.*, 1977). It assumes that different bound peptides in addition to an allo-MHC molecule could produce numerous antigenic determinants that will be recognized by a wider population of cross-reactive T

cells. Recognition could also be initiated due to peptide similarities between the host and the graft, but in spite of that, they appear as foreign due to the conformational modifications induced by the polymorphic residues within the allo-MHC molecule. More recent studies of alloreactive TCRs using crystal structure analysis shows that the bound peptide is involved in numerous close interactions with the alloreactive TCR (Housset *et al.*, 2003; Macdonald *et al.*, 2009). However, both theories are non-mutually exclusive and it is very likely that both models take place during direct allorecognition.

1.3.2 Indirect-pathway

It illustrates the mechanism in which donor MHC molecules are processed and presented by host APC. Among the graft-derived peptides presented by the host APCs are the minor histocompatibility antigens and also peptides from the donor MHC molecules themselves, which are the Major source of polymorphic peptides whereto alloreactive T cells respond. The alloantigens scatter from the graft are mostly processed and presented by self-MHC class II, therefore $CD4^{pos}$ T cells play a Major role in indirect allorecognition.

1.3.3 Semi-direct pathway

Later on it was described a *semi-direct pathway* (Herrera *et al.*, 2004), where recipient APCs trafficking within the graft are able to acquire intact donor MHC/peptide complexes in order to elicit an immune response. The exact mechanism that allows the transfer of intact MHC/peptide complexes between cells is still not fully elucidated. However, this pathway could also explain the described cross-talk between the two main allorecognition pathways (direct and

indirect), whereby host APCs acquire allogenic MHC/peptide complex through MHC transfer in addition to peptides from allogenic histocompatibility antigens, thus a single APC from the host is able to stimulate $CD8^{pos}$ T cells through the direct pathway and also recruit $CD4^{pos}$ T cells via indirect allorecognition.

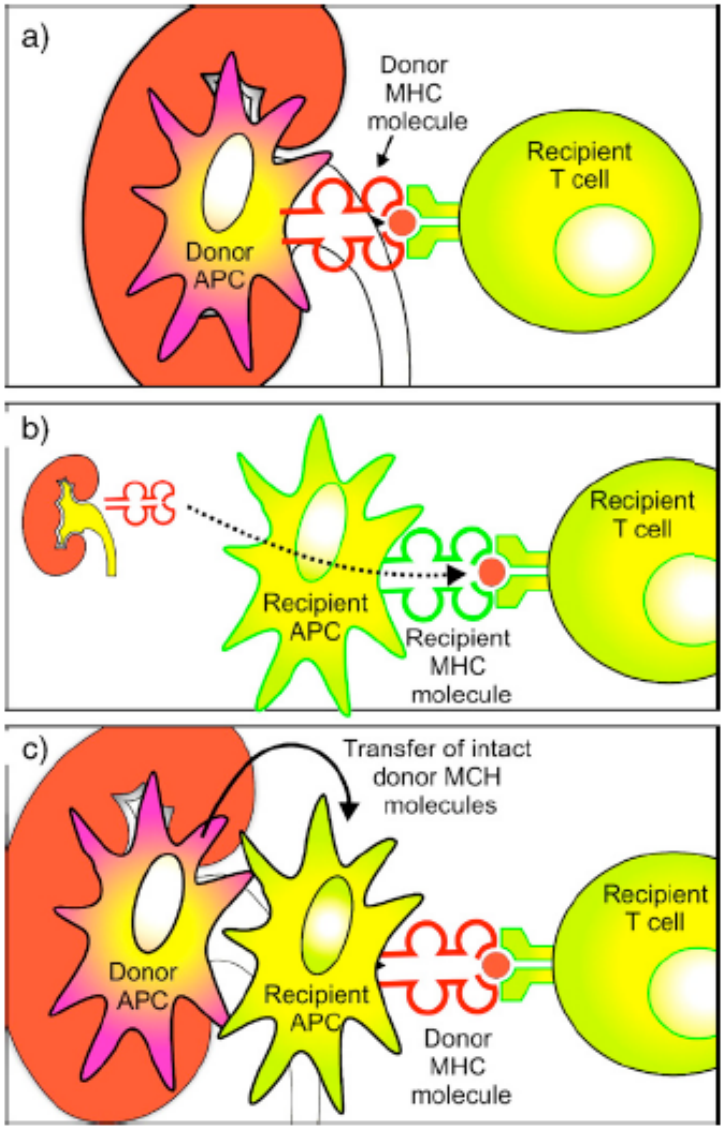


Figure 1.4 Allorecognition pathways. This illustration shows the three mechanisms involved in allorecognition. A) Direct-pathway; B) Indirect-pathway and; C) Semi-direct pathway. Adapted from (Caballero *et al.*, 2006)

The relative clinical contributions from these pathways in graft rejections are not clearly dissected. However growing evidence suggests that direct allorecognition plays a major role during the first phase post-transplant, this mostly in part to the presence of APCs derived from the graft that inherently evoke higher frequencies of alloreactive T cells responding during this initial phase. Conversely, indirect allorecognition is an oligoclonal T cell response, which is mediated by a selected set of alloreactive T cell clones recognizing a few dominant peptides presented by self-MHC (Benichou *et al.*, 1994). This pathway has been mostly associated to chronic rejection, since it requires antigen processing and thus is less rapid than direct allorecognition. As post-transplant time passes, the more prominent indirect allorecognition takes over direct pathway. The relative contribution of semi-direct pathway and graft rejection is as yet unclear.

1.4 Hematopoietic stem cell transplantation (HSCT)

The use of HSCT has become an important modality in the management of hematological malignancies and inherited metabolic disorders (IMD) (Boelens *et al.*, 2010; Hough *et al.*, 2010; Jenq *et al.*, 2010; Nowak, 2008; Socie *et al.*, 2009). The main complication of HSCT is acute or chronic graft versus host disease (GvHD). GvHD is an immunological mediated disease that affects substantially transplant-related morbidity and mortality in HSCT patients. The overall incidence is between 40 to 60%, with approximately a 50% mortality rate.

1.4.1 Acute Graft versus Host Disease

There is currently a three-phase model to exemplify the process that leads to GvHD (Figure 1.5) (Ferrara *et al.*, 2009). *Phase 1* (conditioning/afferent phase): The conditioning regimen used in HSCT initiates the pathogenic process of GvHD. Therefore, the use of myeloblative regimens either with total body irradiation or high-dose of chemotherapy, elicit host tissue damage mainly in the liver, intestinal mucosa between other tissues, through the secretion of inflammatory cytokines (IL-1, TNF-α, GM-CSF and IFN-γ). In addition, this could lead to MHC up-regulation from the host, thus resulting in a wider repertoire of antigens for recognition by alloreactive T cells. *Phase* 2 (induction and expansion): The subsequent presentation of host antigens to donor T cells will evoke proliferation and differentiation towards effector T cells. Donor $CD4^{pos}$ T cells induce GvHD, directed to MHC II molecule disparities, likewise, donor $CD8^{pos}$ T cells will elicit GvHD responding to MHC I disparities. Moreover, disparities in minor histocompatibility antigens (miH) can elicit as well GvHD. Host APCs are essential in this phase, not only for providing host pMHC for recognition, but also via secretion of IL-1 and providing costimulatory signals to alloreactive T cells. *Phase* 3 (effector phase): this complex phase is responsible for the characteristic features of GVHD of end-organ dysfunction and tissue damage. Th-1 preponderance over Th-2 responses constitutively elicits GvHD. Although the cytolytic function of cytotoxic T lymphocytes (CTLs) are paramount in the effector phase of GvHD, NK cell's effector properties have also been implied in GvHD pathology. In general, the cellular damages are caused through these main cellular contact-dependent pathways: TNFα-mediated cytotoxicity, perforin-granzyme B-mediated and Fas-FasL-mediated apoptosis.

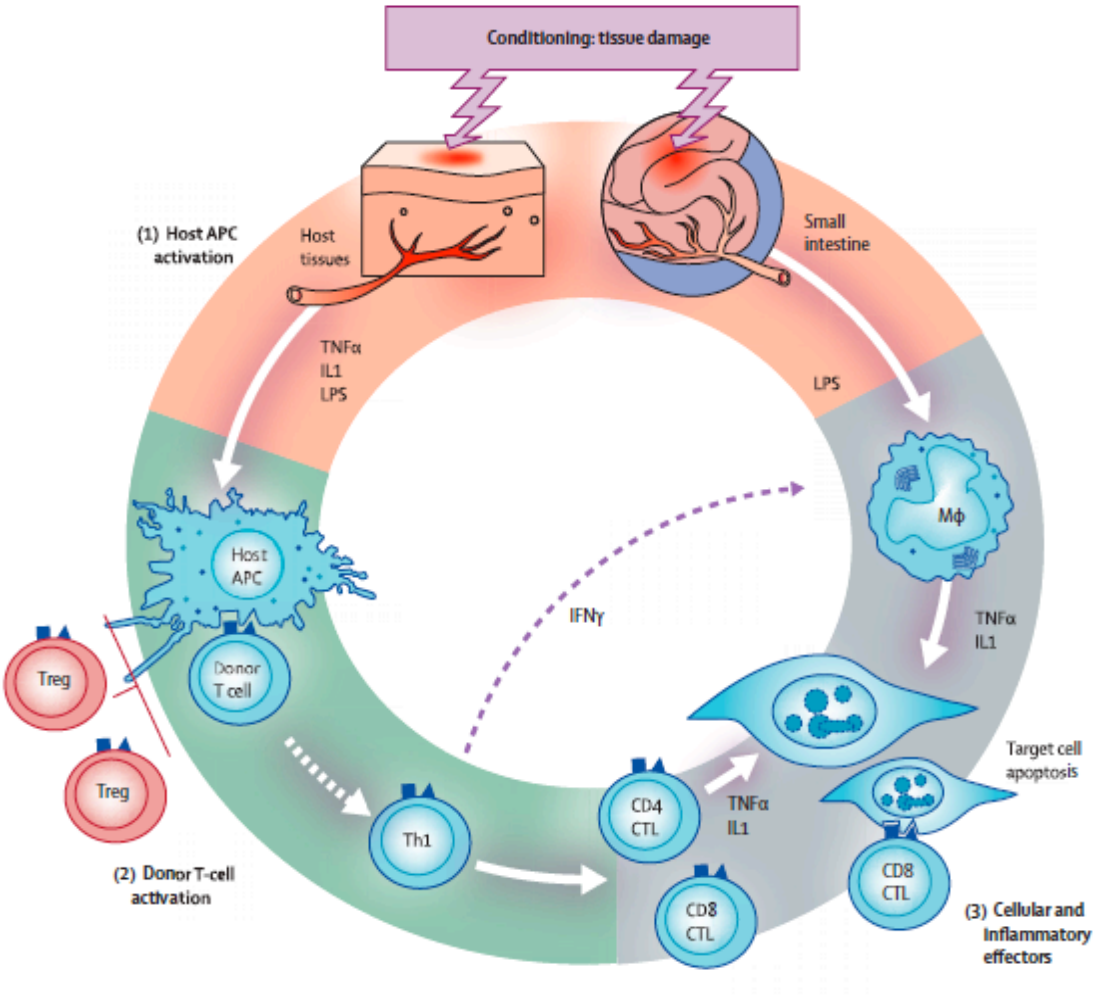


Figure 1.5 Acute GvHD pathophysiology. Adapted from (Ferrara *et al.*, 2009). This illustration exemplifies the three phases of acute GvHD. The main cell subsets as well as the cytokines involved in the process are also shown. In addition it illustrates the therapeutic potential of Tregs within this process.

1.5 Solid organ transplantation (SOT)

The introduction of modern immunosuppressive agents has led to a great success achieving short-term allograft survival (Overall ~92% 1-year graft survival) (Fehr *et al.*, 2004; Lechler *et al.*, 2005) in SOT. However, these agents have not been effective to avoid graft loss due to chronic rejection (Overall ~65% 5-year graft survival). In addition to the correlation between HLA mismatches and graft-survival, alloantigen-independent mechanisms like ischemia-reperfusion injury and growth factors that drive tissue remodeling are also involved in the initiation and perpetuation of chronic rejection. Vascularized grafts like the heart and the kidney are *bona fide* targets for chronic rejection pathology. These grafts slowly develop arterial occlusion as a result of the proliferation of intimal smooth muscle cells and lastly fail due to ischemic damage. In addition and in contrast to HSCT, SOT requires the administration of constant immunosuppression. This leads to important side effects like an enhanced risk of opportunistic infections, cancer, nephrotoxicity, hypertension and cardiovascular disease.

In view of the current results in HSCT and SOT, the common general consensus to address the aforementioned hurdles in both settings is the establishment of immunological tolerance. This approach should prevent GvHD and the development of chronic rejection and will avoid the secondary effects due to the requirement of long-term immunosuppression in SOT.

1.6 Immunological Tolerance

Paul Ehrlich proposed in 1906, that the immune system is programmed to avoid the generation of autoreactive immune responses "*horror autotoxicus"*. Later on, F. Burnet based on his clonal selection theory, demonstrated that *autoreactive* clones are normally deleted at immature stages, however it was considered that some *autoreactive* clones could escape this process. Further on, P. Medawar coined the term "actively acquired tolerance" as: *the state of indifference or non-reactivity towards a substance that would normally be expected to initiate an immunological response* (Billingham et al., 1953; Medawar, 1961). These groundbreaking experiments showed an early window in life wherein the adoption of foreign homologous cells can be accepted and included as part of the self-antigen repertoire and, hence allowing the acceptance of further grafts from the same donor strain.

Immunological tolerance can be subdivided in two non-mutually exclusive mechanisms: *Recessive* and *Dominant*. Recessive tolerance refers to the cell-intrinsic mechanisms that include elimination of self-reactive thymocytes or chronically stimulated peripheral T cell clones by apoptosis or their inactivation through anergy induction. Dominant tolerance is mediated by a specialized subset of cells that restrain pathogenic immune responses. The immunological mechanisms of tolerance to allo-antigens confer great similarity towards the ones involved to self-antigens. However, the greater frequency of alloreactive T cells compared to the lesser repertoire involved to self-antigens becomes a difficult task to outdo in clinical transplantation.

1.6.1 ***Recessive tolerance:*** The thymus plays a major role for the maintenance of self-tolerance. The repertoire of developing thymocytes is purged of cells withholding TCRs with high avidity to peptide-MHC (pMHC) complexes presented in the thymus, process called “thymic negative selection”. In addition, the nuclear factor named autoimmune regulator (Aire), aids this process by controlling the ectopic expression of “tissue restricted antigens” within the medullary thymic epithelial cells (mTECs). Therefore, the two main factors involved in thymic negative selection are: the self or non-self antigen concentration within the thymus and the affinity of the thymocytes TCR that recognizes the antigen. If such conditions occur, T cell deletion can be evoked from double-positive T cells in the thymic cortex or from newly generated single-positive T cells in the medulla (Singh *et al.*, 2006; von Boehmer *et al.*, 2010). This process is important for both $CD8^{pos}$ and $CD4^{pos}$ T cells, since it affects both class I and class II MHC-restricted T cells, respectively. Recessive (also called central) tolerance can be induced experimentally by infusing donor antigens into the thymus. However, this process only achieves transient tolerance due to the lack of continuous administration of donor antigens. Conversely, if donor antigens are administered in a short window early in life or when a replacement of the immune system is attempted using hematopoietic stem cell transplantation based protocols, a continuous supply of donor antigens to the thymus allows life-long negative selection of donor-reactive thymocytes (Fehr *et al.*, 2004; Medawar, 1961).

1.6.1.1 Anergy

Full activation of $CD4^{pos}$ T cells requires the binding of pMHC complexes (signal 1) in addition with co-stimulation provided by APCs (signal 2). However, aberrant activation of the TCR alone in a non-inflammatory scenario can elicit a long-term state of functional unresponsiveness known as *Anergy* (Fathman *et al.*, 2007). This process was first described from in vitro studies, where $CD4^{pos}$ T cells were exposed to pMHC complexes on APCs devoid of B7 molecules. The resulting T cells remain viable whilst unresponsive even to further stimuli by competent APCs. The 2nd signal is required for an efficient TCR-induced transcription of the *il2 gene,* thus anergic T cells are unable to synthesize IL-2, resulting in an abortive proliferative response. Moreover, anergic T cells are unable to secrete inflammatory cytokines such as IFN-γ and TNFα. In addition, T cells can also become anergic when encounter with inhibitory receptors of the CD28 family. The two main inhibitory receptors described are the cytotoxic T lymphocyte-associated Ag-4 (CTLA-4) and programmed cell death-1 (PD-1) (Fife *et al.*, 2008). A CTLA-4 and PD-1 knockout mice, results in uncontrolled multiorgan autoimmunity, suggesting their important role to keep T cells in check. CTLA-4 shows a higher affinity towards CD80 and CD86 on APCs compared to CD28, therefore it is suggested that APCs that express low levels of CD80 or CD86 will preferentially bind to CTLA-4 and therefore dampen the immune response. Costimulatory blockade using CTLA-4-Ig antibody has also been used to efficiently induce tolerance in pre-clinical models. However, T cell deletion and suppression can also be elicited through this approach. Interestingly, the ligands for PD-1, PD-L1 and PD-L2, are not exclusively and functional required to be presented on APCs, since parenchymal tissues can

also expressed these ligands and therefore allow PD-1 activation. It is suggested that CTLA-4-B7 interactions terminate proliferation and promote anergy induction during the initial response to pMHC, whereas PD-1-PD1-ligand interactions might restrain previously tolerized autoreactive T cells that encounter their pMHC complexes within the peripheral tissues.

1.6.1.2 Deletion

Cell death that occurs as a consequence of antigen recognition has been known as activation-induced cell death. This may occur through the activation of a pro-apoptotic protein called Bim. Normal lymphocyte responses elicit anti-apoptotic proteins of the Bcl-2 family, however if T cells encounter antigen in the absence of costimulation, Bim may be activated and elicits death through the mitochondrial pathway. Furthermore, continuous stimulation of T cells can also result in expression of Fas (CD95) and its ligand FasL. Ligation of both receptors leads to the activation of caspase-8 and consequently the induction of apoptosis (Siegel *et al.*, 1990; von Boehmer *et al.*, 2010).

1.6.1.3 Ignorance

One barrier to self/non-self pMHC complex recognition is the physical separation of reactive T cells from the parenchymal cells that express their cognate antigen. This might occur either by the inability of donor antigens (within the graft) to reach the host lymphoid system (no priming), or by the inability of host lymphocytes to reach the graft (no effector function). This mechanism is only achieved in non-vascularized grafts (e.g. corneal allografts); therefore it is unlikely to apply to most of solid organ transplants in humans, since every

vascularized solid organ transplant can be reached by host lymphocytes and passenger leukocytes within the graft traffic to the host lymphoid tissues (Fehr *et al.*, 2004).

1.6.2 Dominant tolerance: Gershon in 1970 first showed the conception of having T cells that not only augmented, but also were able to dampen immune responses and called them “suppressor cells”. Further studies described peripheral immuno-regulatory mechanisms that are also in place withholding allo-reactive cells and allowing a state of tolerance (Dorsch *et al.*, 1975; Roelen *et al.*, 1998; Waldmann, 2008). At present compelling data supports that $CD4^{pos}$ $FoxP3^{pos}$ Tregs represent a known population acting as dedicated mediators of dominant tolerance (Sakaguchi, 2000).

1.7 Types of regulatory T cells

Even though several cells with suppressive capacities have been described (NK, B cells, $CD8^{pos}$, $\gamma\delta$ T cells, DCs, etc), for the purpose of this study I will review briefly the studies on Tr1, Th3, induce $CD4^{pos}$ $FoxP3^{pos}$ Tregs (iTregs) and lastly focus mainly on natural occurring Tregs cells (nTregs), which are the subjects of this study.

1.7.1 Tr1 cells

These specific cells are mainly induced regulatory T cells that have arisen from naïve $CD4^{pos}$ T cells stimulated with tolerogenic dendritic cells in the presence of IL-10 (Foussat *et al.*, 2003; Groux, 2003; Roncarolo *et al.*, 2001). Their main characteristic is high production of IL-10 with lesser secretion of TGF-β,

endowing their capacity to suppress other naïve and memory T cells in vitro and in vivo, this suppressive effect could be reverted by adding IL-10 blocking antibody (Groux, 2003; Roncarolo *et al.*, 2001). Noteworthy, since all the $CD4^{pos}$ T cells including Th1, Th2, Th17 and even nTregs are capable to secrete IL-10 under certain circumstances, Tr1 may represent a certain state of each existing lineage. However, they do not express FoxP3 like nTregs. Therefore, currently there are no specific markers for these cells, thus it is difficult to track them and highly purified them ex vivo, and foremost guarantee their suppressive phenotype stability.

1.7.2 Th3 cells

Some experiments showed that specific non-reactivity to certain antigens can be induced when administered via oral route " Oral tolerance " (Faria *et al.*, 2005). The responsible cells for this effect were reported as $\alpha\beta$T cells restricted to class II with high secretion of TGF-β, IL-10, diminished IL-4 and no secretion of IFN-γ or IL-2 (Chen *et al.*, 2007). In addition, it has been shown that Th-3 cells TGF-β's secretion favors the transcription of FoxP3 and functionality of iTregs (Carrier *et al.*, 2007). At present there are no specific markers available for Th-3 cells, as well as no certainty about their commitment to their suppressive phenotype once they are adoptively transferred in a pro-inflammatory scenario.

1.7.3 Induced $CD4^{pos}$ $FoxP3^{pos}$ Tregs (iTregs)

The induction of $FoxP3^{pos}$ Tregs with suppressive capacity can be elicited from $FoxP3^{neg}$ T cells during homeostatic peripheral proliferation (Curotto de Lafaille *et al.*, 2004). It has also been shown within a transplant tolerance model using a non-depleting CD4 antibody, the conversion from $CD4^{pos}$ naïve T cells into allo-specific $FoxP3^{pos}$ Tregs (Cobbold *et al.*, 2004). Moreover, $CD103^{pos}$ DCs present in the gut and mesenteric lymph nodes are optimal inducers of iTregs through the secretion of retinoic acid and TGF-β (Coombes *et al.*, 2007). As previously mentioned, TGF-β plays an important role in FoxP3 transcription and stability in iTregs (Carrier *et al.*, 2007; Chen *et al.*, 2003; Chen *et al.*, 2007; Horwitz *et al.*, 2003, 2008; Zheng *et al.*, 2002). Therefore, in contrast to nTregs the generation of iTregs, is done in the periphery under various conditions and are dependent of IL-2. Due to the great phenotype similarity between nTregs and iTregs, there is still uncertainty regarding the extent of contribution of each cell subset within an immune response. Nonetheless, it is very likely that wherein a correlation has been found between the presence of Tregs and chronic infections and poor prognosis in solid tumors is mainly due to iTregs (Cavassani *et al.*, 2006; Menetrier-Caux *et al.*, 2009; Piersma *et al.*, 2008). However, nTregs and iTregs may also act synergistically to obtain optimal immune-regulation.

1.7.4 Natural occurring regulatory T cells (nTregs)

The initial studies showing disruption of self-tolerance by mouse neonatal thymectomy, suggested that a thymus-derived cell subset was capable of mediating immune tolerance by withholding other cell subsets (Nishizuka *et al.*, 1969). Neonatal thymectomy between the 2^{nd} and 4^{th} day of life resulted in systemic T cell mediated lesions, which could be reverted by the adoptive transfer of splenocytes or thymocytes from an adult mice (Sakaguchi *et al.*, 1982). Subsequently, S. Sakaguchi in 1995 described a $CD4^{pos}$ T cell population in mice that co-express the IL-2 receptor α chain (CD25), which had the capacity to suppress in a dose dependant way to its counterpart population ($CD4^{pos}$ $CD25^{neg}$)(Sakaguchi *et al.*, 1995). In accordance, a similar population with the same suppressive ability was described in humans (Baecher-Allan et al., 2001).

Mutations in the forkhead box P3 (FoxP3) transcription factor are associated with an autoimmune disorder in human patients called IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) and the murine homologue *scurfy mice* (Bacchetta *et al.*, 2006; Bennett *et al.*, 2001; Chatila *et al.*, 2000; Wildin *et al.*, 2001). The characteristic feature of this disorder is early lymphoproliferative immune-mediated disease affecting a variety of organs and tissues. Eventually, it was shown that FoxP3 is of great importance for Tregs development and functionality (Fontenot et al., 2003). However, later on, it was also shown that FoxP3 expression could also be upregulated transiently in effector T cells that confer no suppressive function (Morgan *et al.*, 2005;

Roncarolo *et al.*, 2008). Nonetheless, FoxP3 is the most accepted marker for nTreg characterization in a steady state of no inflammation.

These regulatory T cells have been coined as natural occurring self-antigen driven emigrants from the thymus and normally constitute around 4 to 10% from the $CD4^{pos}$ T cells (Ito et al., 2008; Sakaguchi et al., 2008). $FoxP3^{pos}$ cells are detected with increased frequency since $CD4^{pos}$ $CD8^{pos}$ double-positive to $CD4^{pos}$ $CD8^{neg}$ single-positive stages (Fontenot *et al.*, 2005c). Recently, it was suggested that nTreg selection is facilitated by TCRs that endow affinities towards self-peptides falling within a range between positive selection of conventional $CD4^{pos}$ T cells and negative selection of high-affinity self-reactive T cells (Wing *et al.*, 2010). Therefore, FoxP3 development is difficult to be explained solely on TCR specificity. This notion supports the studies that have shown an important TCR overlap between conventional $CD4^{pos}$ $CD25^{neg}$ T cells and nTregs in humans (Pacholczyk *et al.*, 2006). Besides TCR signaling contribution for nTreg development, it has been shown a marked decrease in frequencies of Tregs in CD28-deficient and CD80/CD86-deficient mice (Tai *et al.*, 2005). Therefore, the costimulatory pathway has an essential cell-intrinsic role in nTregs differentiation.

In addition, IL-2 is also essential for nTregs differentiation, since IL-2 or IL-2Rα chain deficient mice, exhibit a marked decrease of $FoxP3^{pos}$ thymocytes (Fontenot *et al.*, 2005a). Furthermore, activated STAT5, downstream of IL-2 receptor signaling, regulates FoxP3 transcription (Burchill et al., 2007; Wuest et al., 2008). Therefore, IL-2 is paramount for Tregs differentiation and phenotype

stability. In contrast to in vitro studies, Tregs have shown the capacity of proliferating in vivo (Klein et al., 2003). Recently it has also been shown that the activation of the transcription factors Smad3 and NFAT, through the presence of TGF-β, is necessary for the histone-acetylation pattern in the *Foxp3 5'* enhancer region, suggesting their involvement in nTreg development (Tone *et al.*, 2008).

It has been shown that induction of Tregs towards non-inherited HLA maternal antigens (NIMAs) are developed in early life *in utero* (Mold et al., 2008). These Tregs are maintained throughout life preserving their potent suppression ability against maternal antigens. However, during lifetime nTregs suffer phenotype changes, mainly starting with a naïve phenotype and eventually becoming central memory (Santner-Nanan et al., 2008). In reference some studies have explained that a subset of Tregs pool is maintained throughout life by homeostatic proliferation generated from highly differentiated memory $CD4^{pos}$ T cells (Vukmanovic-Stejic et al., 2006). This, in theory would allow having enough Treg cell numbers throughout life to maintain self-tolerance and counteract any possible deleterious immune response towards the host. nTregs also express the glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR). This receptor has been shown to have increased expression upon T-cell activation (Zhan et al., 2004), and it is thought to play a key role in dominant immunological self-tolerance. In accordance, signalling through this receptor abrogates nTregs suppressive capacity (McHugh *et al.*, 2002; Nocentini *et al.*, 2005; Shimizu *et al.*, 2002).

1.8 Mechanisms of suppression

For the purpose of this study I will only focus on the mechanisms of suppression reported from $CD4^{pos}$ $FoxP3^{pos}$ Tregs (nTregs and iTregs). Most of the studies in vitro have shown that Tregs suppression is cell-contact dependent (Thornton *et al.*, 2000). Although a more current consensus suggests that the activation process is cell-contact dependent, and once activated, the suppressive mechanisms will vary depending of the site, target cell and evolving factors of the immune response in hand (Vignali et al., 2008). The variety of Tregs suppressive mechanisms can be summarized into those that directly target T cells and those that primarily target APCs (Figure 1.6 A and B) (Shevach, 2009).

1.8.1 Mechanisms that target conventional T cells

Cytolysis: Some studies have shown that Tregs expressed granzyme A and that in conjunction with perforin and adhesion to CD18, they can mediate target-cell killing (Grossman et al., 2004). Moreover it was shown a lack of suppressive ability in Tregs from mice with granzyme-B-deficiency (Gondek et al., 2005).

IL-2 consumption: Initial studies showed that IL-2 is also essential for Tregs suppressive function (Thornton *et al.*, 2004a). Others have shown that Tregs compete for IL-2 secreted from effector T cells, resulting in effector T cell apoptosis (Pandiyan *et al.*, 2007).

Cytokine mediated: Several studies in vivo have also shown that different cytokines like IL-10, TGF-β and more recently IL-35 are involved in Tregs suppression ability. Although, there is still some controversy whether these cytokines are secreted by nTregs or iTregs (Vignali et al., 2008).

IL-10: A population characterized as $CD4^{pos}$ $FoxP3^{pos}$ $ICOS^{pos}$, has shown to mediate suppression through contact with dendritic cells in an IL-10 dependent manner (Ito *et al.*, 2008). Allergen-specific Tregs adoptively transferred to an asthma mouse model were able to control the disease (Roncarolo et al., 2001). This effect was reverted by blocking-IL-10 antibody. However, this effect was also seen when administered IL-10 deficient Tregs. In addition, it has been shown that the tumor microenvironment induces FoxP3+ T cells that mediate suppression through secretion of IL-10, affecting tumor immunosurveillance (Strauss *et al.*, 2007). Moreover, IL-10 production by Tregs are essential for preventing immunopathology in mouse IBD models (Asseman et al., 1999).

TGF-β: From current studies it has been shown that Tregs can mediate suppression either by soluble or membrane-tethered TGF-β. TGF-β secreted from Tregs has been shown in different scenarios like chronic infections, allergy, IBD models and tumors (Huber *et al.*, 2006; Nakamura *et al.*, 2001). Moreover it was shown that membrane-tethered TGF-β Tregs can control $CD8^{pos}$ T cells infiltration in a diabetes mouse model (Green et al., 2003).

IL-35: Recently it was discovered a new regulatory cytokine: IL-35, which is a member of the IL-12 family. It was shown a preferential expression of IL-35 in $FoxP3^{pos}$ Tregs and not in resting or activated effector cells. In addition it was shown that ectopic expression of IL-35 in naïve T cells conferred them regulatory activity (Collison et al., 2007) (Figure 1.6 A).

1.8.2 Mechanisms directed towards APCs

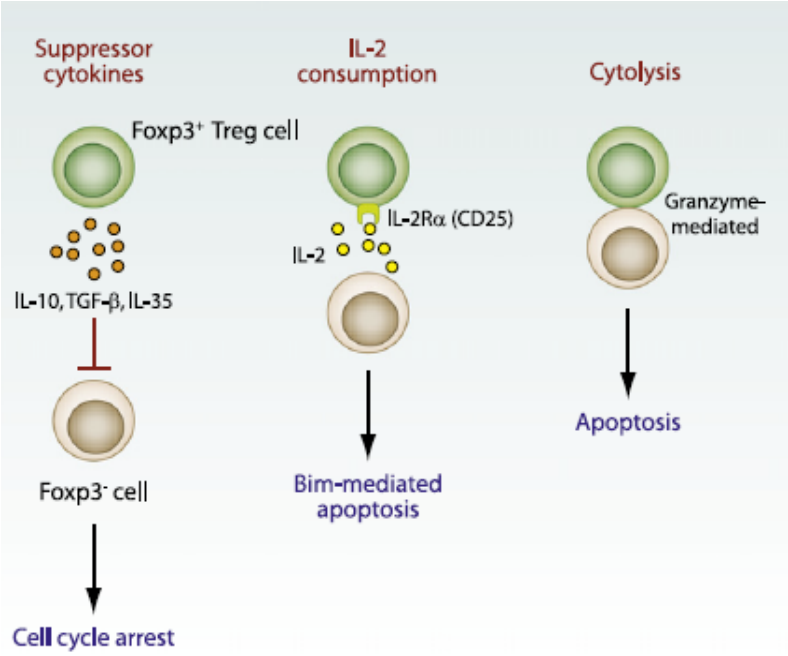
L-selectin (CD62L) acts as a "homing receptor" for leukocytes to enter secondary lymphoid tissues via high endothelial venules. CD62L-mediated lymphocyte recirculation is required for the proper distribution of both naïve and effector T cells between sites of inflammation and secondary lymphoid tissues (Grailer et al., 2009). In accordance, it has been demonstrated in a mice GvHD model, that only $CD62L^{high}$ Tregs interfere with the activation and expansion of GvHD effector T cells within the secondary lymph nodes (Taylor et al., 2004).

It has also been demonstrated using intravital miscroscopy that Tregs highly interact with dendritic cells (DCs) in vivo (Tang *et al.*, 2006). Tregs constitutively express CTLA-4, which can elicit inhibitory signals to DCs, thus, diminishing effector T cell activation. CTLA-4 signal can activate indoleamine-2,3-dioxygenase (IDO), which generates the immunosuppressive mediator kynurenin (Grohmann et al., 2002). Signaling through CTLA-4 also promotes the nuclear localization of Foxo transcription factors, which hampers the expression of genes encoding IL-6 and the phosphorylation of the tumor necrosis factor (Dejean *et al*., 2009), hence, blocking the cytokine-mediated inflammatory cascade. Secondly, it has been shown that Tregs expressed LAG3, which is a CD4 homologue that will bind to the MHC class II molecules blocking DC's maturation (Huang et al., 2004). Likewise, it has been recently discovered that neuropilin (Nrp-1), receptor for class III semaphorins and a coreceptor for vascular endothelial growth factor (VEGF), promotes longer interactions between Tregs and immature DCs, thus inhibiting interactions between naïve T effector cells and DCs (Sarris *et al*., 2008).

Lastly, in the immune system, extracellular ATP functions as an indicator of tissue destruction and also favors APCs maturation. CD39 is the dominant ectoenzyme that has the capacity to hydrolyze ATP or ADP into AMP. CD39 expression among Tregs has been associated with their suppressive function by inducing the immunoinhibitory molecule adenosine (Borsellino et al., 2007; Deaglio et al., 2007; Fletcher et al., 2009).

In summary we can say that Tregs have a wide range of suppressive mechanisms. The context in which the immune response evolves will dictate the role of Tregs and their mechanisms of suppression (Figure 1.6 B).

A)



B)

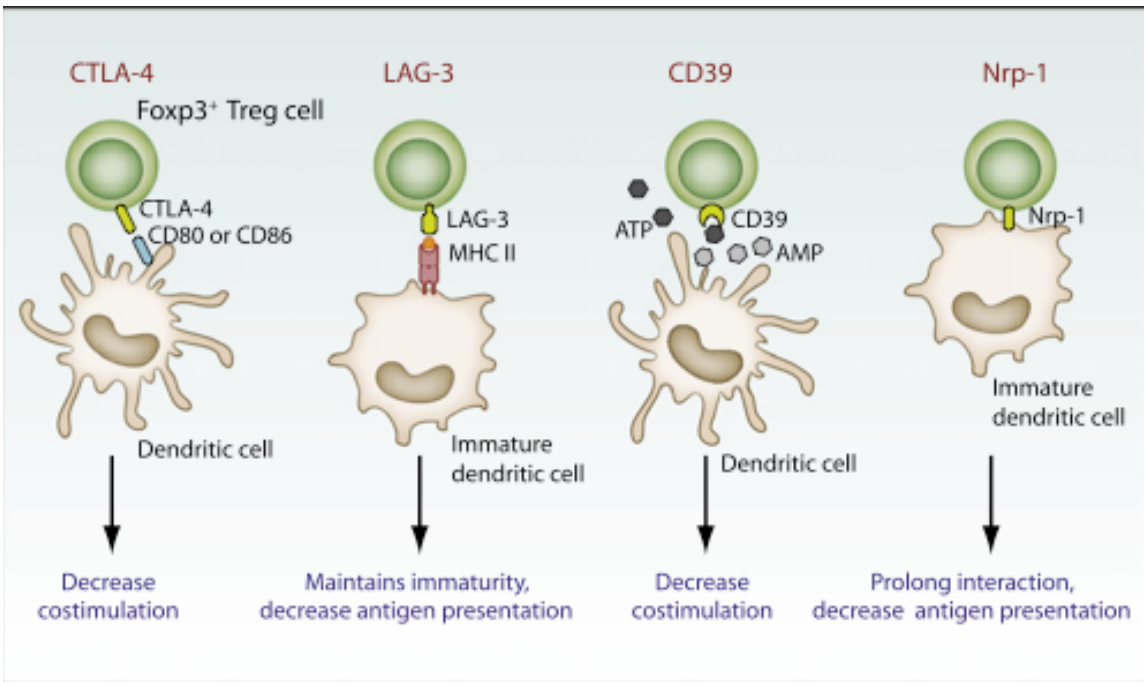


Figure 1.6 Mechanism of suppression of CD4^{pos} FoxP3^{pos} Tregs. This illustration shows the mechanisms of suppression of Tregs and divide them in context of their target cell subset; A) Directed towards conventional T cells and B) directed towards APCs. These illustrations are adapted from (Shevach, 2009).

1.9 Clinical evidence and applications

The initial identification of FoxP3 mutation in IPEX patients was solid evidence to support the existence and clinical relevance of nTregs (Bacchetta *et al.*, 2006; Bennett *et al.*, 2001; Chatila *et al.*, 2000). Interestingly, these patients confer a similar frequency of $CD4^{pos}$ $CD25^{high}$ T cells in comparison to healthy controls. However, the suppressive capacity of this Tregs is diminished and also the capacity of their conventional T cells to produce IL-2 and IFN-γ upon activation (Bacchetta *et al.*, 2006). Subsequently, compelling data has evolved around Tregs association with poor prognosis in solid organ tumors (Li *et al*., 2007; Perrone *et al*., 2008). It has been proposed that tumor cells induce Tregs that will create a tolerogenic microenvironment around the tumor that could restrain effector T cells response against them. Current cancer therapies for these tumors are focusing either on depleting Tregs or targeting key factors involved in Tregs mechanisms of suppression (Curiel, 2008). In concordance with these reports, it has also been shown correlation between higher Tregs frequency and chronic immunopathology by some pathogens (Cavassani *et al*., 2006; Hasenkrug, 2003).

Recently, it has been proposed that recent thymic emigrants (RTE) naïve $CD4^{pos}$ T cells ($CD4^{pos}$ $CD45RA^{pos}$ $CD31^{pos}$) are important to prevent autoimmunity (Kohler *et al.*, 2008). In concordance, it has been demonstrated in multiple sclerosis patients that a decrease of $CD45RA^{pos}$ $CD31^{pos}$ Tregs could be associated with the immunopathology of these patients (Haas et al., 2007). Moreover, data has been reported about defective Tregs in type1 diabetes

(T1D) and rheumatoid arthritis (RA) patients (Brusko *et al.*, 2008a; Nadkarni *et al.*, 2007).

In the area of solid organ transplantation, to my knowledge only one study has been able to correlate Tregs with good graft survival (Eljaafari et al., 2006). Conversely, in HSCT settings, it has been shown an association between GVHD and a reduced ratio between effector and Tregs cells (Matthews *et al.*, 2009; Rezvani *et al.*, 2006). Current data on cord blood used as a source for HSCT has suggested that the combination of naïve Tregs and naïve effector T cells, could be partly responsible for the lower degree of GvHD reported (Chang *et al*., 2005; Hippen et al., 2008). In concordance, several studies have shown the potent suppressive ability of cord blood naïve Tregs *(Bresatz et al., 2007; Ito et al*., 2008; Porter *et al*., 2006; Santner-Nanan *et al*., 2008). Lately, evolving data has supported that $CD45RA^{pos}$ Tregs represent the most homogenous population with superior suppressive capacity (Hoffmann *et al*., 2006b; Seddiki *et al*., 2006b).

New strategies are currently ongoing to achieve adequate Treg cell numbers for cellular immuno-therapy with high purity and potent suppressive capacity (Baecher-Allan, 2006; Bresatz *et al*., 2007). Since FoxP3 is an intranuclear marker it cannot be used for viable cell isolations. Furthermore, CD4 and CD25 gating strategies are not specific enough to isolate pure Tregs from adults. Recent sorting techniques have been described using $CD25^{high}$ $CD127^{low}$ gate on $CD4^{pos}$ T cells, this shows a 90% correlation with $FoxP3^{pos}$ cells ex vivo and in vivo (Liu *et al*., 2006; Seddiki *et al*., 2006a). Most of the studies on Tregs isolation methods for clinical purposes have recommended multiple step protocols, mostly CD8, CD19 and most recently CD49d depletion (Kleinewietfeld

et al., 2009; Peters et al., 2008; Riley et al., 2009). Nevertheless, low purity (<60% FoxP3^{pos}) continues to be an issue for stepping into bigger scale models and clinical trials.

Additionally, Tregs only constitute between 4 to 10% of peripheral CD4^{pos} T cells, thus, expansion techniques have been created needing 3 to 4 weeks time of culture with >200 folds increase (Brusko *et al.*, 2008b). Most of the expansion protocols have shown the need to add rapamycin to prevent outgrowth of effector contaminating cells (Battaglia et al., 2006). This mainly because of the higher level of contaminating effector cells (~40%) using current GMP isolation methods. Therefore, insufficient yield and low purity are still the main drawbacks to achieve a high purity product with clinical grade standards for cellular immunotherapy (Hoffmann et al., 2006a; Peters et al., 2008; Wichlan et al., 2006).

1.10 Objective of this study

The objective of this study is to test the "Third party approach" using CB Tregs to suppress alloresponses. In order to address this, the first objective is to fully characterize CB Tregs in comparison to adult Tregs. Secondly, optimize a one step CD25 Ab selection protocol for cord blood Tregs, which could be translated to large-scale isolations with high purity and clinical grade standards. Thirdly, test their suppressive potency in comparison with host adult Tregs. Fourthly, test the feasibility of mixing *"Pooling"* naive Tregs from cord blood units and evaluate their suppressive effect in vitro. The hypothesis is that by specifically pooling naive Treg cells from cord blood we could maintain their individual proliferative capacity as well as their naïve repertoire. Since these cells will be considered "third party", it will be of great importance to have multiple repertoires to increase the chances of TCR engagement in vivo and thus activation.

CHAPTER 2

MATERIAL AND METHODS

2.1 Introduction

The study described in this thesis focuses on the isolation of $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ T cells from cord blood mononuclear cells (CBMCs) employing magnetic particles coupled with specific antibodies. The results and translation from the two-step protocol recommended by the manufacturer employed in adult peripheral mononuclear cells (PBMCs) to a single-step protocol for CBMC's will be shown in the relevant result chapter. This is to highlight the new optimized protocol that enable the study of highly purified $CD4^{pos}$ $CD25^{pos}$ $CD127^{low}$ T cells using a practical and reproducible one-step method. Results obtained during the optimization of different techniques and functional assays will be included within this chapter rather than in the results chapter, this with the only intention to focus more on the measurements regarding the actual effect *in vitro* or *in vivo* of $CD4^{pos}$ $CD25^{pos}$ $CD127^{low}$ T cells from cord blood rather than the results of the preliminary experiments.

2.2 Samples and healthy donors

CB units were collected from normal and cesarean full-term deliveries from the Barcelona cord blood bank with prior written consent and approval by the ethical committee. CB units were collected in sterile bags containing 25ml of citrate-phosphate-dextrose (CPD) using conventional techniques and

transported to the Anthony Nolan Research Institute (ANRI) in temperature between 4 and 21°C.

To collect peripheral blood from healthy controls a qualified doctor under prior written consent and approval by the internal ethical committee took a range between 20 to 60ml of peripheral blood from members of the Anthony Nolan staff and was processed immediately as described in Section 2.3.

2.3 Processing of blood samples

CBU upon arrival to the ANRI were diluted 1:1 with transport medium (TM): RPMI 1640 (Lonza, Belgium) supplemented with trisodium citrate at a final concentration of 3.3% (w/v) and mercaptoethanol (BDH Biochemicals) at a final concentration of 5μM and used within 60hrs after collection. CBMC's were isolated by density gradient by overlaying 35ml of cord blood onto 15ml Ficoll-Paque Premium (GE Healthcare, UK) in 50ml Falcon tubes and centrifugedd at 2,200 rpm (no break) for 35mins. Ficoll-Paque Premium with a density of 1.073 g/ml has been optimized to isolate mononuclear cells from CB units and is recommended in accordance with GMP (Good Manufacturer Practice) for the manufactured of cell therapy products. Centrifugation of cord blood over this reagent results in the formation of layers containing different cell types; the bottom layer contains erythrocytes, the layer immediately above contains mostly granulocytes and the mononuclear cells will be sitting just at the interface between plasma and the Ficoll. CBMC's were carefully harvested and washed twice in RPMI 1640.

PBMCs from healthy volunteers were isolated with the same principle. Although, peripheral blood was not diluted with TM and Lympholyte (Cedarlane Laboratories, Canada) was used for the density gradient separation. In addition the initial centrifugation step was set up at 2,000rpm for only 20 minutes (no brake).

2.3.1 Cell enumeration and viability

Cell counts and viability were performed by dye exclusion method. Therefore 10μl of the cell suspension were stained with an equal amount (10μl) of 0.4% trypan blue (BDH). Viable cells (non-stained) were counted in a haemocytometer (Neubaur Chamber, Weber) under a phase contrast Leica DM LB microscope (Meyer Instrument). Percentage of non-viable cells was also recorded with each measurement. Fresh samples always had >95% viability.

2.3.2 Cryopreservation of cells

The vast majority of the cellular experiments in this study were carried out using fresh blood samples. Cells that needed to be stored for use at later time points were cryopreserved in a pre-cooled freezing mix solution: 90% heat-inactivated fetal calf serum (hiFCS) and 10% dimethyl sulfoxide (DMSO) (BDH). Therefore 1ml aliquots containing a minimum of $1x10^7$ cells were transferred to 1.5ml cryotube vials (Nunc) and immediately place in freezing containers (Sigma Aldrich) at -80°C for 24hrs for a recommended uniform cooling rate of 1°C per minute from ambient temperature (by indirect immersion in an isopropyl alcohol bath) before storage in liquid nitrogen. A

standard protocol was made to thaw the cells and placed into culture. Cryotubes were rapidly warm-up in a water bath at 37°C. Followed by transfer (drop wise) of the cell suspension into 10ml of pre-warmed RPMI 1640 supplemented with 10% heat-inactivated human AB serum and 1U/ml penicillin and 1μg/ml streptomycin (all Bio Whittaker). This supplemented medium will be hereafter referred as culture medium. Subsequently, the cells were washed twice using centrifugation (with brake) at 1,600 rpm for 10 minutes. Prior cell enumeration and viability measurement, cells were incubated in culture medium for at least 2hrs at 37°C/5%CO_2 in a humidified incubator (IG 150,Jouan). The recovery rate was >75% with a viability >85%.

2.4 Fluorescent cell surface staining and analysis

Cells stained for cell surface markers using directly conjugated antibodies were visualized using flow cytometry. This is a commonly used technique based on the principles of light excitation, light scatter and emission of different fluorochromes, which generate specific data about particles or cells as they flow within a fluid stream through a beam of light. In general the properties that can be measured are cell relative size, granularity and fluorescence intensity. A FACSCalibur flow cytometer (Becton Dickinson) was used in this study.

Cell surface staining was carried out using between $5x10^4$ to $3x10^5$ cells allocated in 96 well V bottom plates (Nunc) and centrifuged at 4°C/ 1700rpm for 6min (with brake) followed by two washes with pre-cooled PBS supplemented with 1% hiFCS. Subsequently, cell surface staining was done

adding 47μl (this taking into account a wet pellet of 3μl) of previously done antibodies cocktails (*i.e* CD4-APC, CD25-PE, CD127-Percp) at the recommended concentrations for 15 minutes in the dark at 4°C. This was followed by two more washes in the same manner. Samples were either immediately processed and acquired on the flow cytometer and analyzed using FlowJo software Version 6.0 (Tristar) or fixed in PBS/1 % (w/v) paraformaldeheyde (PFA) (BDH) and stored at 4°C in the dark until acquisition within 24hrs.

Antibodies directed to different cell surface markers were purchased from BD Pharmingen, Ebiosciense, Miltenyi and R&D as detailed in table 2-1. Each antibody was titrated to acquire an optimal staining, as it will be described in section 2.4.2.

Table 2-1 List of surface markers antibodies used in flow cytometry

Marker	Format	Concentration used	Clone	Company
CD3	PE	1/10	SK7	BD
CD4	PerCP/APC	1/25, 1/10	SK3,RPA-T4	BD, eBioscience
CD8	PerCP	1/50	SK1	BD
CD25	APC,PE	1/25,1/100	2A3,4E3	BD, Miltenyi
CD31	APC	1/50	9G11	R&D
CD39	APC	1/25	TU66	BD
CD45RA	PE	1/100	HI100	BD
CD45RO	APC	1/10	UCHL1	BD
CD62L	FITC	1/50	Dreg-56	BD
ICOS	PE	1/25	DX29	BD
LAP	PE	1/50	27232	R&D
CTLA-4	PE	1/25	BNI3	BD
GITR	FITC	1/25	110416	R&D
CXCR3	APC	1/5	1C6/CXCR3	BD
CD127	FITC/PerCP-Cy5.5	1/10	eBioRDR5	Ebioscience
HLA-DR	FITC,PerCP	1/25 both	L243	BD
CD69	PerCP	1/50	L78	BD
LAG-3	PE	1/25	AALE02	R&D
CD103	PE	1/50	Ber-ACT8	BD
CD132	PE	1/25	AG184	BD

2.4.1 Fluorescent intracellular staining

A wide range of intracellular markers as well as the recognition of intracellular cytokines that are involved in immunological events can also be clearly depicted in a single cell level using flow cytometry. One of the main markers for proper study of Tregs is the intra-nuclear transcription factor FoxP3. Intracellular staining for FoxP3 was carried out as recommended by the manufacturer (Ebioscience). Previously stained cells with cell surface markers (*i.e.* CD4-APC, CD25-PE and CD127-PerCP-Cy5.5) were fixed and permeabilized with Fix/Perm buffer for 30 minutes at 4°C in the dark, followed by two washing steps using permeabilization buffer (both Ebioscience). Cells were then incubated with the proper FoxP3 antibody at the following concentrations: FoxP3-FITC (1/20) or FoxP3-PerCP (1/50), for 30 minutes at 4°C in the dark. In addition, cells were also stained with the proper isotype-matched control in the same manner. Cells were washed twice with permeabilization buffer and resuspended in 120μl of pre-cooled PBS 1% hiFCS until acquisition within 24hrs. Representative staining of Tregs characterization from PBMCs using the recommended markers are shown in Figure 2-1.

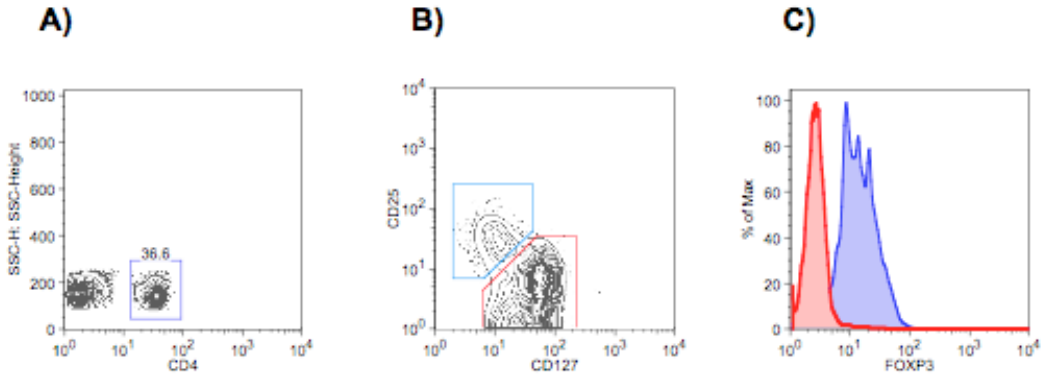


Figure 2.1 Example of Tregs characterization by flow cytometry: A) Gate on $CD4^{pos}$ cells from lymphocyte gate, **B)** Tregs gate described as $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ (blue) cells and Effector cells described as $CD4^{pos}$ $CD127^{high}$ (red). **C)** Histogram showing their corresponding expression of FoxP3.

The same protocol was used for intracellular cytokine staining. If the tested cells were also stained for FoxP3, Ebioscience' reagents were used (since optimal FoxP3 staining is strictly dependent on the manufacturer protocol and reagents and does not jeopardized intracellular cytokine staining). BD reagents were used when no FoxP3 staining was required in the experiment. After proper stimulation with polyclonal stimuli (described in section 2.5.2), cells were incubated for at least 5hrs (5 to 7hrs) with 0.5μl BD Golgi-stop (aka monensin, final concentration of 2.0μM). Later on cells were harvested, washed and stained (with the proper concentration of each antibody) for 30 minutes at 4°C as previously described. Additional cells were also stimulated and stained with the proper isotype-matched antibodies. Table 2-2 describes the monoclonal antibodies used in this study for intracellular staining.

Table 2-2 List of antibodies used for intracellular staining

Marker	Format	Concentration used	Clone	Company
FoxP3	FITC,PerCP-Cy.5.5	1/20,1/100	PCH101, 236A/E7	Ebioscience
IFN-γ	APC	1/50	B27	BD
IL-17a	PE	1/50	TC11-18H10	BD
IL-4	PE	1/50	MP4-25D2	BD
IL-10	APC	1/50	JES3-19F1	BD

2.4.2 Titration of directly conjugated antibodies

For optimal staining and proper identification of the wanted cell subtypes, each antibody used was titrated using a standard protocol unless otherwise recommended by the manufacturer. Dilutions from 1/5 to 1/100 were done using the previously mentioned protocols for cell surface and intracellular staining in section 2.4 and 2.4.1 respectively. The optimal dilution was chosen using the following criteria: 1) Proper identification of the positive and negative population (when applicable) 2) no shift from the negative population mean fluorescence intensity (MFI) towards the positive population (compared to non-stained cells and 3) Ratio among the positive and negative populations within the range of what has been reported in previous studies or stated by the manufacturer. A representative titration using intracellular and cell surface markers is shown in Figure 2-2.

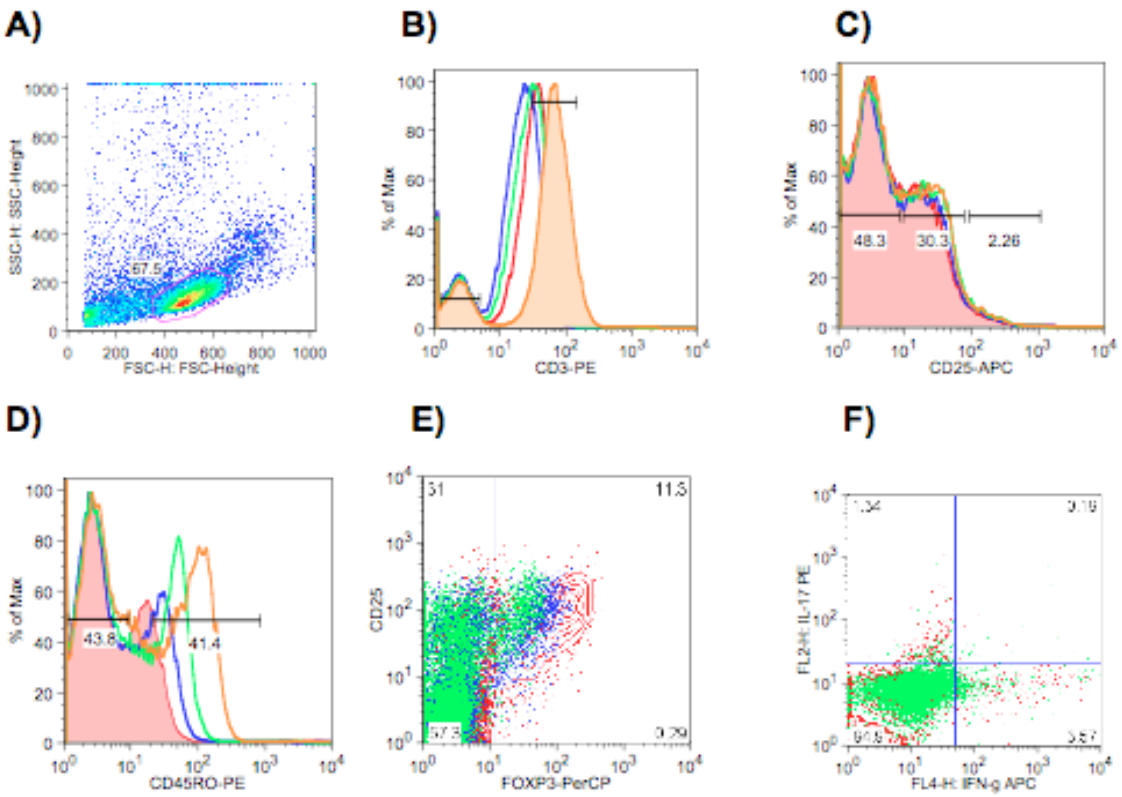


Figure 2.2 Representative example of titration of cell surface and intracellular antibodies: A) Lymphocyte gate shown by FSC and SSC. Histograms, shows the titration of surface markers **B)** CD3-PE, **C)** CD25-APC and **D)** CD45RO-PE) gated from lymphocyte gate. **E)** Overlay color graphs plotting on CD25 (*y-axis*) versus FoxP3-Percp (*x-axis*) using different antibody concentrations (1/100, 1/50 and 1/25). **F)** Stimulated PBMCs stained with IFN-γ-APC and IL-17a-PE (1/100 and 1/50). Positive gates were done using isotype-matched controls.

2.4.3 Compensation and isotype controls

Since flow cytometry uses different fluorochromes that emit a specific range of wavelengths, some overlap can be seen (FL1⇔FL2, FL-2⇔FL3, FL3⇔FL4). This spectral overlap was manually compensated using non-stained cells, and stained cells with each of the different fluorochromes (FITC, PE, PerCP and APC) to clearly identify the positive and negative populations, either unstained cells or isotype-matched controls were used

each time to properly delineate the wanted population, an example of the use of isotype controls is demonstrated in Figure 2-3.

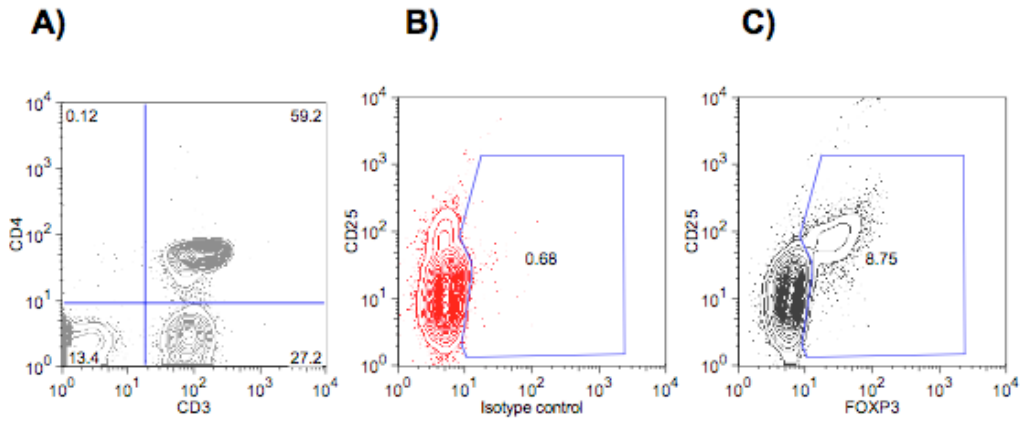


Figure 2.3 Example of the use of isotype control for FoxP3 gating. A) PBMCs were gated on $CD3^{pos}$ $CD4^{pos}$ cells. **B)** In red cells were stained with isotype matched control, and plotted against CD25. **C)** Gate was set up to cover <1% of the events and use it on the cells stained with FoxP3.

2.5 Functional assays

2.5.1 Mixed lymphocyte cultures (MLC)

A one-way MLC was done to exemplify an allo-response *in vitro*. To get a reproducible allo-response and also to minimize the number of cells required for each functional assay, titration experiments were done to establish the optimal amount of cells needed as "responders" and "stimulators". This was of specific interest for the suppression assays, since it was important to have a reproducible *in vitro* model that would allow us to titrate the number of Tregs needed to suppress an allo-response. Different cell numbers (10^4, $2x10^4$, $5x10^4$, $1x10^5$ and $2x10^5$ cells) were placed either as "stimulators" or

"responders" in both directions (n=4). A representative experiment is shown in Figure 2-4.

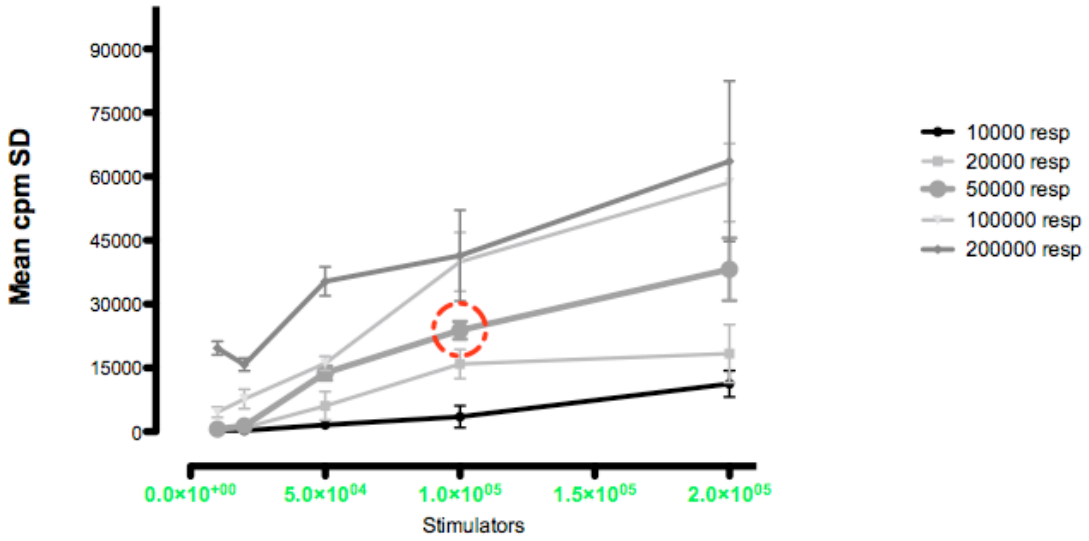


Figure 2.4 A representative experiment of MLC titration (n=4): Graph shows on the *X*-axis the number of irradiated PBMCs (stimulators) used to stimulate the different numbers of responder cells. Results are shown as mean c.p.m. SD. Red circle shows the combination selected for further experiments.

The combination of $5x10^4$ "responders" cells stimulated with 10^5 irradiated "stimulator" cells showed less variability and reproducible allo-response without over-crowding the system in 4 independent experiments. This combination was selected and used for further MLCs and set up as follow: PBMCs or previously isolated adult $CD4^{pos}$ $CD25^{neg}$ T cells were resuspended in culture medium at 10^6 cells/ml. A total of $5x10^4$ cells were used as "responders" and stimulated with mismatched $1x10^5$ PBMCs that were fully irradiated with 10,000 Rad in a Cesium γ-irradiator for 7 minutes. Cultures were placed on 96 well U bottom culture plates (SARSTEDT, USA) in a final volume of 200μl. Each experiment was set up in triplicates (unless mentioned otherwise) and incubated at 37°C/5% CO_2. After 5 days of culture, each well was pulsed with 1μCi [H^3] thymidine

(Amersham) for 16 to 18hrs. Cellular proliferation was quantified by thymidine uptake and incorporation into genomic DNA from dividing cells and measured using a liquid scintillation counter 1450 Microbeta (Perkin Elmer Wallac). Results were expressed as mean counts per minute (c.p.m.). Suppression was calculated using the following formula:

% suppression = 1- [c.p.m. effector with Tregs/ c.p.m. effectors alone] x100**.**

2.5.2 Polyclonal stimulation assay

In order to have a model non-dependent of antigen presenting cells (APC) and antigen non-specific, a polyclonal stimulation assay was carried out using soluble anti-CD3 human antibody (clone: HIT3a, BD) and soluble anti-CD28 human antibody (clone CD28.2, BD). *In vitro* T lymphocyte stimulation is extensively used to study T cell function and for expansion protocols. Different mitogenic molecules such as lectins and monoclonal antibodies promote polyclonal proliferation whereas specific antigens yield an oligoclonal response. Antibodies specific for the TCR-CD3 complex provide an initial activation signal (aka Signal 1), but proliferation is dependent on a costimulatory signal, usually provided by cross-linking of CD28 (aka Signal 2).

In order to optimize this method, previously isolated $5x10^4$ $CD4^{pos}$ T cells (detailed in section 2.6) from CBMCs and PBMCs were incubated with different concentrations of anti-CD3 and anti-CD28 either alone or in combination. Autologous feeder cells (irradiated PBMC or CBMC) from both $CD4^{pos}$ T cells sources in combination with different concentrations of soluble anti-CD3 human antibody were also tested to elicit activation. As a positive control, $CD4^{pos}$ T cells

from CB and adult PBMC were also stimulated with 10^5 irradiated mismatched PBMCs. Each combination was set up in triplicates in 96 flat bottom culture plates (Becton Dickinson, New Jersey) and incubated at 37°C/ 5%CO_2 in a final volume of 200μl. After 5 days of culture, cells were pulsed with 1μCi [H^3] thymidine (Amersham) for 16 to 18hrs. Cellular proliferation was quantified by thymidine uptake as previously described using a liquid scintillation counter 1450 Microbeta (Perkin Elmer Wallac). Results were expressed as mean counts per minute (c.p.m.). A representative experiment (n=3) is shown in Figure 2.5.

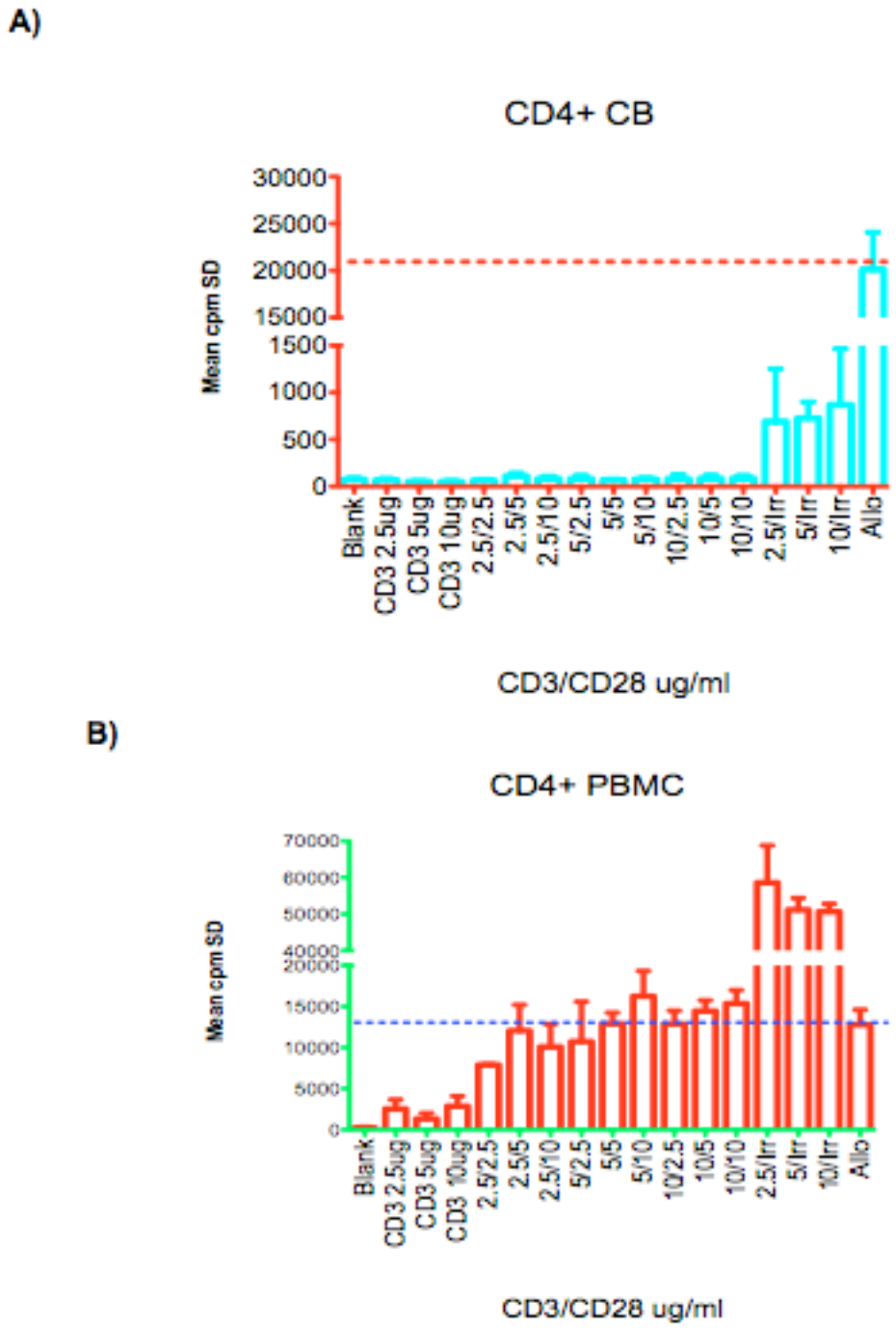


Figure 2.5 A representative experiment of polyclonal stimulation of CD4^{pos} T cells from CB and PBMCs (n=3). A) CB CD4^{pos} and **B)** adult CD4^{pos} cells were stimulated with soluble anti-CD3 human antibody at 2.5, 5 and 10μg/ml alone or in combination with anti-CD28 human antibody (at 2.5,5 and 10μg/ml) or with autologous irradiated feeder cells (10^5 cells). Both CD4^{pos} T cells were also stimulated with mismatch (*allo*) irradiated PBMCs as a positive control. Dotted line delineates the mean proliferation of the MLR used as positive control.

CB $CD4^{pos}$ T cells showed less proliferation capacity compared to $CD4^{pos}$ T cells from adult PBMCs. In addition, CB $CD4^{pos}$ T cells were only able to respond appropriately to mismatched irradiated PBMCs, whereas adult $CD4^{pos}$ T cells responded to every combination of anti-CD3/anti-CD28 and anti-CD3+ autologous feeder cells in a reproducible fashion. Taking these results, every further suppression assay described in this thesis will be done using only adult $CD4^{pos}$ T cells or total PBMC as responder cells. Polyclonal stimuli for suppression assays will be done using soluble anti-CD3 at 2.5μg/ml and anti-CD28 at 5μg/ml.

2.5.3 Staining with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFDASE)

Staining with carboxyfluorescein diacetate succinimidyl ester (CFSE) has become, a routine technique in many laboratories since its first description by Lyons and Parish in 1994. CFSE has the ability to stably label molecules within cells, with each cell division resulting in a sequential dilution of fluorescence. Initially, CFDASE is non-fluorescent, due to the presence of two acetate groups, but these compounds enable the dye freely across the plasma membrane (Hawkins et al., 2007; Quah et al., 2007). Once inside the cell, the acetate groups are removed by intracellular esterases. This will yield the highly fluorescent CFSE trapped inside the cells. The fluorescent conjugates that persist within the cell will be diluted between daughter cells following cell division, which allows lymphocyte proliferation to be monitored by flow cytometry.

Optimal staining of the studied cells was accomplished using the following protocol: a total of 1 to $2x10^6$/ml cells in PBS 5% hiFCS were stained with 2.5μM CFSE from a 5mM stock solution prepared in DMSO (Invitrogen Molecular Probes) at room temperature (RT) in the dark for 5 minutes. CFSE stained cells were centrifugedd at 1700 rpm for 5 minutes and washed three times with 10ml PBS 5% hiFCS. After washing steps, cells were resuspended at 10^6 cells/ml in culture medium and incubated at 37°C/ 5%CO_2 incubator for subsequent stimulation. Figure 2.6 shows an example of CFSE proliferation after time course of polyclonal stimulation in correlation with [H^3] thymidine uptake.

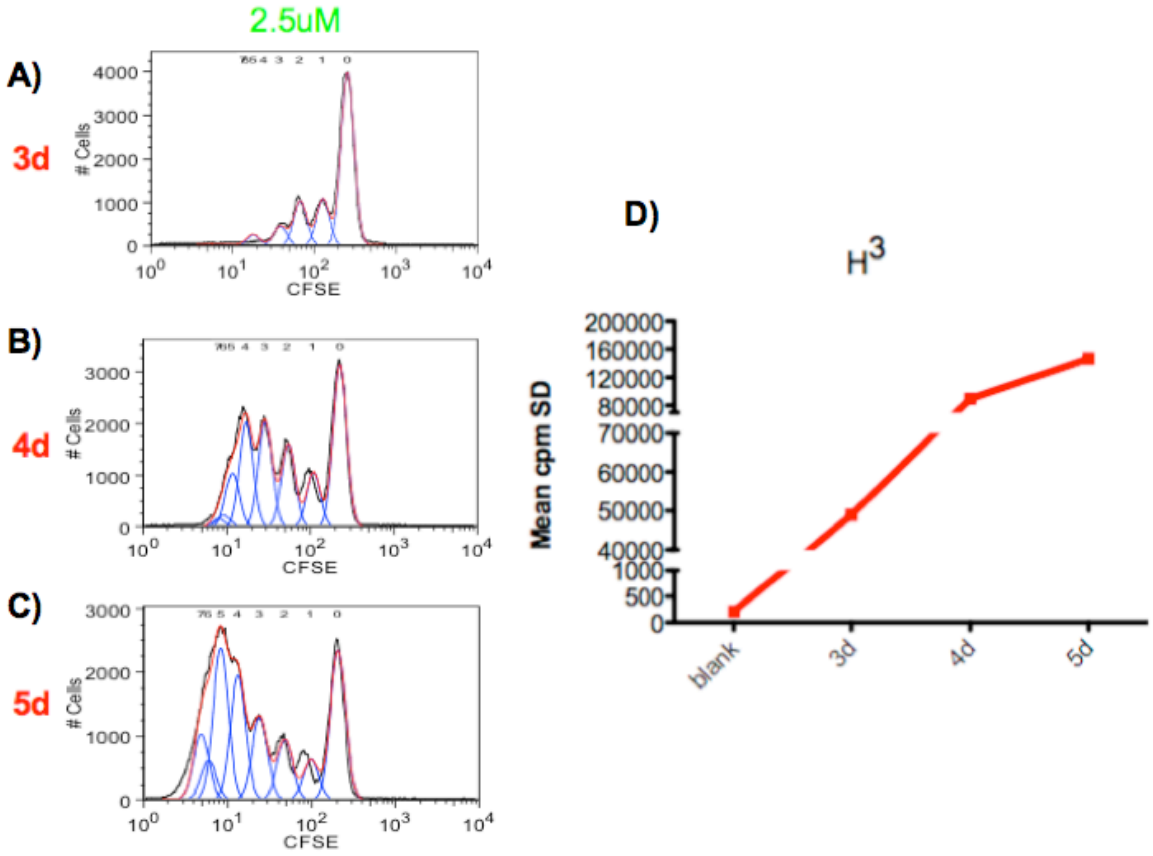


Figure 2.6 Polyclonal stimulation of CD4^{pos} T cells measured with CFSE. CD4^{pos} T cells were stained with 2.5μM CFSE and stimulated with soluble anti-CD3/CD28 human antibodies. Time course of **A)** 3, **B)** 4 and **C)** 5 days were set up in triplicates. Gates were done on lymphocytes set up by FSC and SSC. **D)** Parallel triplicates were also set for [H^3] thymidine uptake at the same time points.

CFSE assays were carried out for 4 days of culture when polyclonal stimulation was used and for 6 days when allo-response was measured. After culture, cells were spun at 1,700rpm for 5 minutes and washed twice in pre-cooled PBS supplemented with 1% hiFCS. Subsequently, cells were stained for surface markers and if needed eventually intracellular markers as described in section 2.4 and 2.41.

At last, cells were then immediately acquired on a FACSCalibur flow cytometer (BD). Suppression was calculated as follows:

% suppression = %$CFSE^{pos}$ proliferating cells with Tregs/ % $CFSE^{pos}$ proliferating cells without Tregs x 100.

2.5.4 Trans-well suppression assay

To measure if Tregs were able to suppress in a cell-cell contact independent matter, MLCs (described in section 2.5.1) were done using 96 or 24 trans-well plates with inserts of 0.4μm pore size (Corning, Costar). Tregs resuspended at 10^6cells/ml in culture medium were added in a 1:2 ratio (Tregs:Effectors) either in the lower chamber or in the upper chamber. When placed in the upper chamber they can be with or without irradiated PBMCs used as stimulators at a 1:2 ratio (Tregs:Irradiated PBMCs). Cultures were placed on a final volume of 200μl (96 well plates) or 500μl (24 well plates) of culture medium and set up as triplicates. After 5 days of culture, each well was pulsed with 1μCi [H^3] thymidine (Amersham) for 16 to 18hrs. Cellular proliferation was quantified by thymidine uptake and measured using a liquid scintillation counter 1450 Microbeta (Perkin Elmer Wallac). Results were expressed as mean c.p.m. Suppression was calculated using the same formula described in section 2.5.1.

2.6 Isolation of $CD4^{pos}$ $CD25^{pos}$ T cells from PBMCs using magnetic beads

Isolation of untouched $CD4^{pos}$ T cells was done through negative selection using indirect magnetic labeling of non-$CD4^{pos}$ T cells as recommended by manufacturer (Miltenyi Biotec, Germany). Briefly, PBMC or CBMC were resuspended with 90μl per 10^7cells of chilled isolation buffer (1x PBS with 0.5%

BSA or hiFCS and 2mM EDTA) and stained with 10μl per 10^7cells of biotin-antibody cocktail (which include anti-CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and CD235a) for 10 minutes at 4°C. Subsequently, a second layer was done using anti-biotin microbeads at 20μl per 10^7cells and incubated for 15 minutes at 4°C. After incubation, cells were washed with 2ml of isolation buffer and centrifuged at 1400rpm for 10 minutes at 4°C. After discarding supernatant, cells were resuspended in 500μl of isolation buffer and passed through a LD column placed on a QuadroMACS isolation magnet (both Miltenyi). Two washing steps were carried out with 1ml of isolation buffer, and the effluent was collected containing the unlabeled pre-enriched $CD4^{pos}$ T cell fraction. This fraction was used in some experiments that required only $CD4^{pos}$ T cells. This method achieved >95% purity for $CD3^{pos}$ $CD4^{pos}$ T cells. For CD25 positive isolation, the cell suspension was counted (section 2.3.1), spun down and resuspended in 90μl per 10^7 cells of isolation buffer and stained with 10μl per 10^7cells of directly conjugated CD25 microbeads (Miltenyi) and incubated at 4°C for 15 minutes. After a washing step with 5ml of isolation buffer, cell suspension was resuspended in 500μl of isolation buffer and passed through a LS column placed on the isolation magnet and rinsed with 2ml of isolation buffer. The negative fraction was collected and used as effector cells in functional assays. The CD25 positive fraction was collected after extracting the LS column from the magnet and rinse with 1ml of isolation buffer applying the plunger. This step was repeated to achieve better purity. After the second passage through an LS column, the cells were counted and resuspended in culture medium and

incubated at 37°C/ 5%CO_2 upon their use. Figure 2-7 shows a representative isolation of $CD4^{pos}$ $CD25^{pos}$ T cells from adult PBMC.

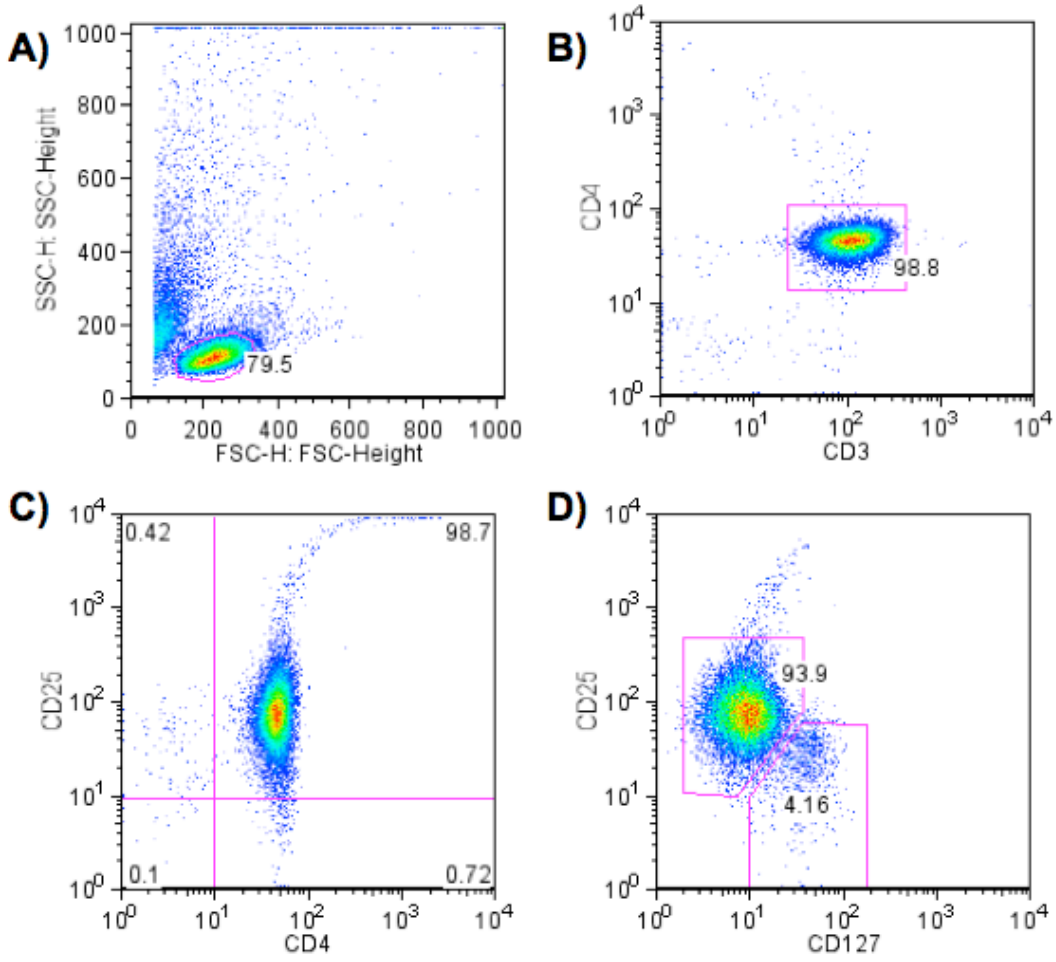


Figure 2.7 Characterization, of a CD4 enrichment and CD25 positive isolation from PBMC. Color plots showing gating strategy to characterize $CD4^{pos}$ $CD25^{pos}$ isolation from PBMC. **A)** Lymphocyte gate was set up from FSC and SSC. **B)** $CD3^{pos}$ $CD4^{pos}$ double positive population is taken from lymphocyte gate. **C)** $CD4^{pos}$ T cells are plotted against CD25 or **D)** using CD25 versus CD127 gating strategy. Tregs are depicted as $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ cells and effector cells as $CD4^{pos}$ $CD127^{high}$.

2.7 IL-17 measurement by ELISA

Noteworthy, IL-17 measurement from cell-culture supernatant was done in collaboration with Guy's Hospital at their facilities using their standard protocol: ELISA for human IL-17 was carried out using the Duo-Set ELISA kits from R&D (R&D systems). Nunc Maxisorb™ plates were coated overnight at RT with 100μl/well of anti-IL-17 at a final concentration of 4 μg/ml in PBS. Plates were washed three times with 0.05% Tween 20 (Sigma) in PBS and blocked for one hour at RT with 300ml/well freshly made, filtered, 1% bovine serum albumin (BSA) (Sigma) in PBS. Plates were washed three times as before and dry up before addition of 100μl/well of samples and standards in duplicate. Standard curves were prepared by serial two-fold dilutions of the manufacturer's standards in 1% BSA v/v in PBS from a top concentration of 1000pg/mL to 15.63pg/ml and one concentration of 0pg/ml (diluent alone). Plates were incubated for two hours and washed three times as before. A total of 100μl/well of the detection antibody was added at a final concentration of 75ng/ml of IL-17 in 1% BSA v/v in PBS and incubated for two hours at RT. Plates were washed three times with 0.05% Twean/PBS and 100μl manufacturer's streptavidin-HRP (horseradish peroxidase) diluted 1 in 20 (in 1%BSA/PBS) was added to each well and incubated for 20 minutes. Plates were again washed three times and dried up before the addition of 100μl substrate solution (TMB) (Zymed, San Francisco, CA) to each well. A further incubation for 20 minutes at RT in the dark was followed by acidic termination of the reaction through addition of 50ml 0.5M sulphuric acid (H_2SO_4) to each well. Optical density at 450nm was measured on a Bio-Tek EL800 automatic plate reader (Wolf Laboratories, UK).

The concentration of IL-17 was calculated from standard curves constructed from the optical densities of the known concentrations.

2.8 Determination of HLA type

Healthy volunteer samples and cord blood units DNA-based HLA typing were performed at the Histocompatibility Laboratories at the Anthony Nolan Research Institute (ANRI). HLA class I and class II genotyping was performed using sequence-specific oligonucleotides (PCR-SSO), sequence-specific primers (PCR-SSP), or nucleotide sequencing.

2.9 Analysis

Statistical analysis and graphs were carried out using Prism 5 software for MAC OS X Graphpad package. In general, *t* test and one-way ANOVA were used when applicable to identify significance within functional assays and isolation

CHAPTER 3

CB TREGS CHARACTERIZATION.

Introduction 3.1

A wide variety of markers have been ascribed to Tregs phenotype and functionality since the reborn studies of regulatory T cells (Sakaguchi *et al*., 1995). Currently, FoxP3 is the most accepted marker for a proper identification of nTregs in a non-inflammatory state (Seddiki *et al*., 2006a). Strong correlation between the IL-2 receptor α chain (CD25) and FoxP3 expression has been widely described since the identification of FoxP3 in these cells (Fontenot *et al.*, 2003; Thornton *et al.*, 2004a). However, currently it is widely known that adult $CD4^{pos}$ $FoxP3^{pos}$ T cells represent a small percentage from overall $CD4^{pos}$ $CD25^{pos}$ T cells, and also, latest studies have shown a scarce population of $FoxP3^{pos}$ cells among the $CD4^{pos}$ $CD25^{neg}$ T cells (Fontenot *et al.*, 2005b; Miyara *et al*., 2009). Moreover, experiments intended to discover a more homogenous Treg population, had demonstrated that $FoxP3^{pos}$ cells are mainly $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ (IL-7 α receptor) whereas effector cells are $CD4^{pos}$ $CD127^{high}$ (Liu *et al*., 2006; Seddiki *et al*., 2006a). Compelling data has demonstrated that $CD25^{pos}$ $CD45RA^{pos}$ Tregs represent the most homogenous population among the overall nTreg pool (Hoffmann *et al.*, 2006b; Putnam *et al.*, 2009). More recently (Miyara *et al*., 2009) classified nTregs into two main populations; resting Tregs (rTregs) $CD45RA^{pos}$ $CD25^{pos}$ $FoxP3^{low}$ and activated Tregs (aTregs) $CD45RA^{neg}$ $CD25^{high}$ $FoxP3^{high}$. Both populations showed stable Treg phenotype upon activation and proliferative capacity *in vitro and in vivo*. In summary,

regardless of the gating strategy, a level of FoxP3 expression is always assessed. Therefore, gating on FoxP3^{pos} from total CD4^{pos} T cells is the first step recommended to properly identify nTregs. Further characterizations should be measured from the double positive population (CD4^{pos} FoxP3^{pos} T cells). Noteworthy, this approach is only recommended in a steady state *in vivo* or freshly isolated *ex vivo* (Roncarolo *et al.*, 2008). For characterization of Tregs in an inflammatory setting or post expansion cultures *in vitro*, measurements at the epigenetic level, specifically, methylation at the FoxP3 locus is required for a proper Treg identification (Baron et al., 2007; Polansky et al., 2008).

Results 3.2

The purpose of this chapter is firstly to characterize Tregs from CB in comparison to adult Tregs and, secondly, to measure the expression of different markers that have been shown to be important for Treg phenotype and functionality.

3.2.1 Treg frequency is similar in adult PBMCs and CBMCs.

As shown in Figure 3-1, gating on FoxP3^{pos} cells, CB Tregs represented between 5 to 9% from overall CD4^{pos} T cells (n=10). No major difference was seen when CD25^{high} CD127^{low} gate was used (5-8%). Likewise, gating on FoxP3^{pos} cells, adult Tregs represented between 6 to 10% from CD4^{pos} T cells (n=10). However, a slightly lower amount of FoxP3^{pos} cells was depicted using CD25^{high} CD127^{low} gate (4 to 8%).

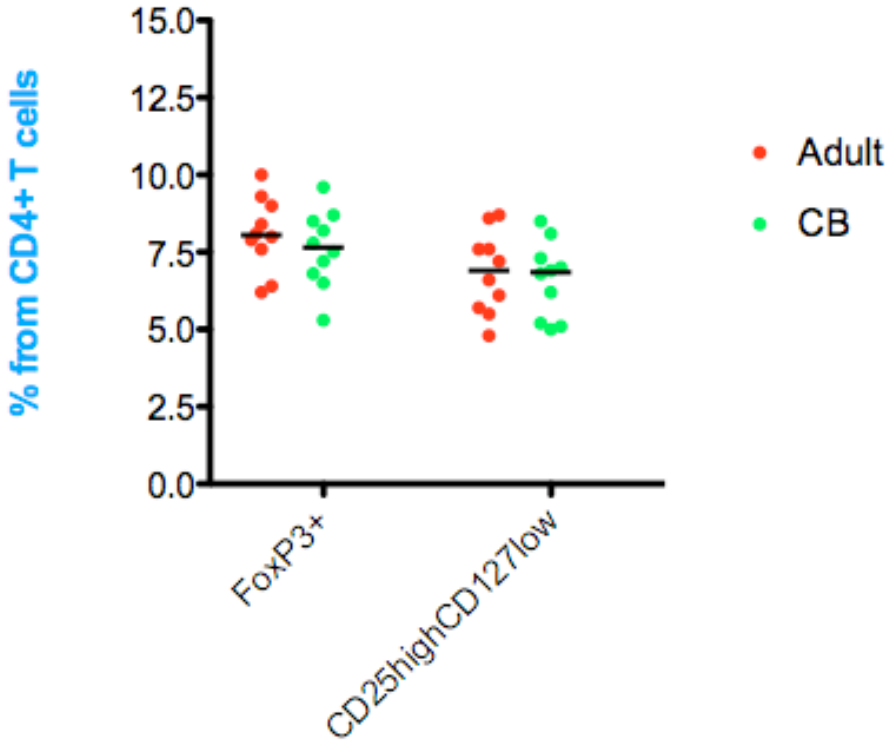


Figure 3.1 Tregs identification from adult PBMC and CB (n=10). Tregs were characterized as FoxP3^{pos} cells and CD25^{high}CD127^{low} gate from CD4^{pos} T cells. Red dots represent characterization from adult PBMCs and green dots from CBMCs. Results are shown with scatter plots, **black line** depicts the median value.

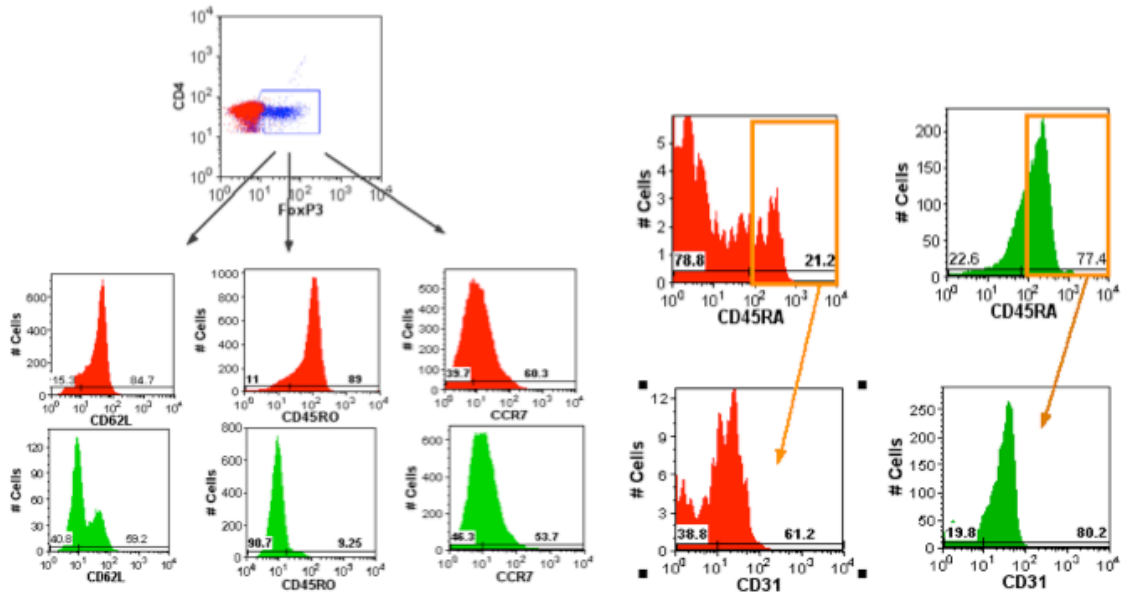
3.2.2 CB Tregs are mainly naïve (CD45RA^{pos}).

The main difference between CB Tregs and adult Tregs is their naïve phenotype. Gating on CD4^{pos} FoxP3^{pos} T cells, CB Tregs are 89% CD45RA^{pos} (range 68 to 92%), in contrast adult Tregs are 18% CD45RA^{pos} (range 8 to 40%; Figure 3-2). Accordingly, CB Tregs are 9% CD45RO^{pos} (range 8 to 19%) and adult Tregs 80% CD45RO^{pos} (range 48 to 91%). Furthermore, the expression of the lymphocyte chemokine receptor CCR7, which is also used to further characterize naïve T cells (Sallusto *et al.*, 2000; Takada *et al.*, 2009), showed no clear difference when measured between CB and adult Tregs (range 65 to 82%

and 56 to 72%, respectively). In contrast, a higher proportion of adult Tregs expressed the lymphocyte adhesion molecule L-selectin (CD62L) compared to CB Tregs (mean 79%, range 68 to 84%; mean 55%, range 38 to 57%, p=0.0001, respectively). CD62L-mediated lymphocyte recirculation is required for the proper distribution of both naïve and effector T cells between sites of inflammation and secondary lymphoid tissues (Grailer et al., 2009).

To further characterize the $CD45RA^{pos}$ Treg population, CD31 expression was measured to identify the recent thymic emigrant Treg (RTE) population (Haas et al., 2007; Junge et al., 2007). Noteworthy, there is a higher proportion of $CD45RA^{pos}$ Tregs in CB compared to adult $CD45RA^{pos}$ Tregs. Nonetheless, CB showed a higher proportion of $CD31^{pos}$ subset compared to adult (mean 80±4 *vs* 64±3.3, p=0.008, respectively). In summary, the substantial difference depicted between CB and adult Tregs was mainly the naïve phenotype of the former. In addition, the majority of $CD45RA^{pos}$ $FoxP3^{pos}$ CB Tregs are $CD31^{pos}$, which will be considered as RTE Tregs that constitutively expressed a wide TCR repertoire.

A)



B)

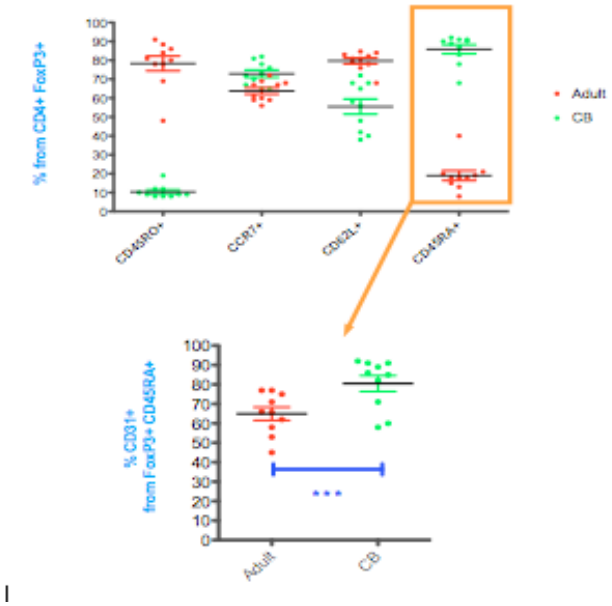


Figure 3.2 CB and adult Tregs characterization. A) The upper histograms show a representative gating strategy for phenotype characterization of CB (depicted in green) and PBMCs (depicted in red) Tregs. **B)** The upper graph depicts expression of the mentioned markers from $CD4^{pos}$ $FoxP3^{pos}$ T cells from **CBMCs in green** (n=10) and **adult PBMCs in red** (n=10). The lower graph shows the level of CD31 expression from $CD4^{pos}$ $FoxP3^{pos}$ $CD45RA^{pos}$. Results are shown as mean values ± SEM. Statistical analysis was done using an unpaired two-tailed *t* Test (***p=<0.001).

As previously mentioned, currently it has been shown that Tregs can be characterized in two main subsets. aTregs which are $CD25^{pos}$ $FoxP3^{high}$ $CD45RA^{neg}$ and rTregs characterized as $CD25^{pos}$ $Foxp3^{low}$ $CD45RA^{pos}$ (Miyara et al., 2009). Using this gating strategy and as expected, CB Tregs are mainly rTregs with a lower proportion of aTregs (mean 82%, range 79 to 86%; mean 13%, range 11 to 16, respectively). Conversely, adult Tregs are mainly aTregs, with a scarce population being rTregs (mean 76.6%, range 69 to 83%; mean 17.6%, range 15 to 20%, respectively) (Figure 3.3).

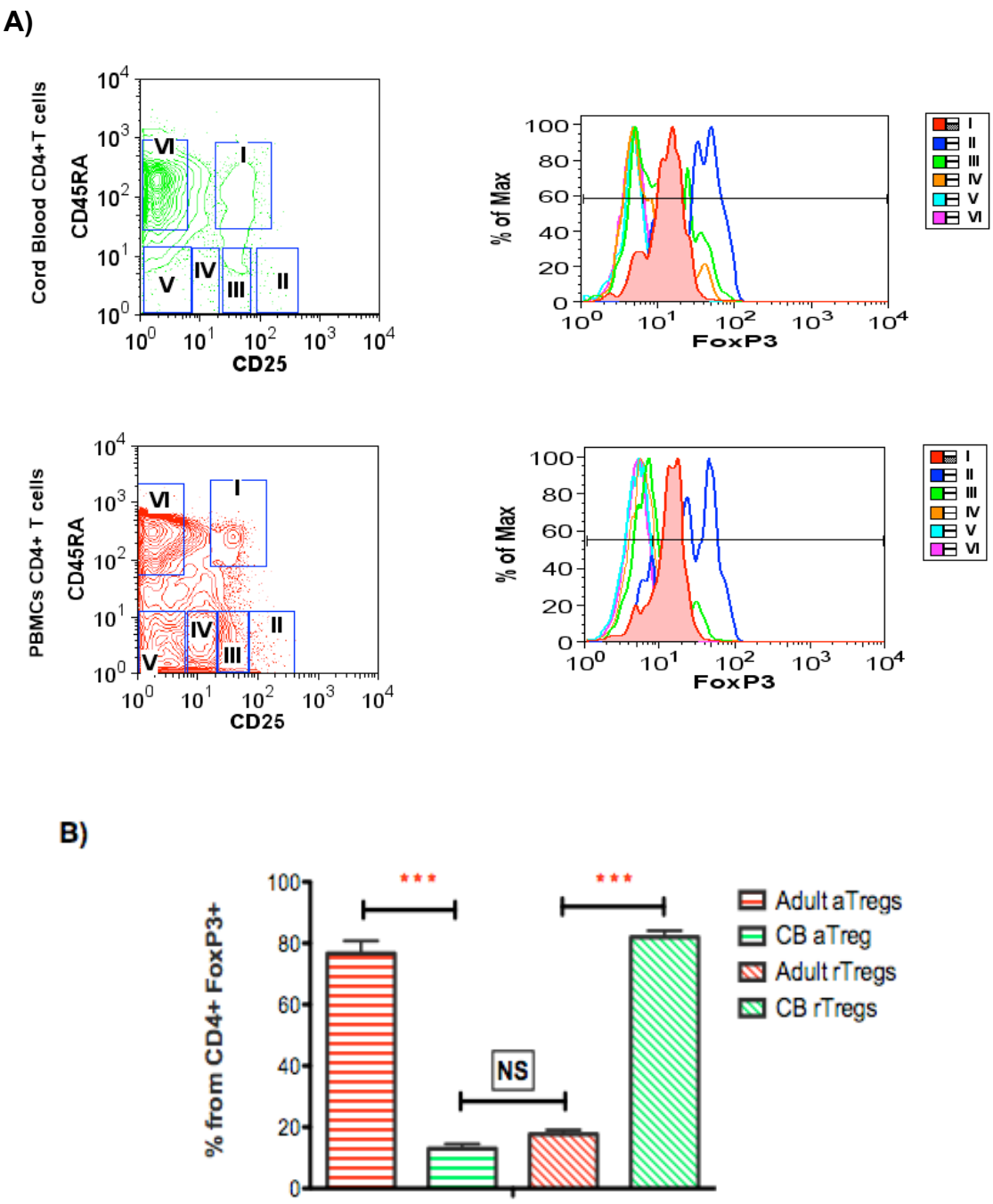


Figure 3.3 Representative rTregs and aTregs characterization from CB (n=3) and adult PBMC (n=3). A) The upper counter plots with their corresponding histogram overlay (right) shows a representative characterization of **CB** and **adult** $CD4^{pos}$ T cells using CD25 and CD45RA expression. Overlay histograms shows the level of FoxP3 expression of each population depicted (I to VI). **B)** Lower graph shows the proportion of rTregs and aTregs from complete $CD4^{pos}$ $FoxP3^{pos}$ cells. Populations II and III were considered as aTregs and population I as rTregs (Miyara et al., 2009). Results are shown as mean ±SEM (***p=<0.0001).

3.2.3 CB Tregs constitutively express a wide variety of markers related to Treg functionality.

In adult Tregs, a wide repertoire of markers has been correlated to their suppressive properties (Brusko *et al.*, 2008b). CB $CD4^{pos}$ $FoxP3^{pos}$ (n=8) Tregs constitutively express most of these markers (Figure 3.4). Noteworthy, whilst Tregs are on a steady state in a non-inflammatory environment, most of these markers are expressed in lower levels. In contrast, once Tregs get activated, they start up-regulating most of these markers as it will be shown in details, when Treg activation pattern is described (Chapter 6).

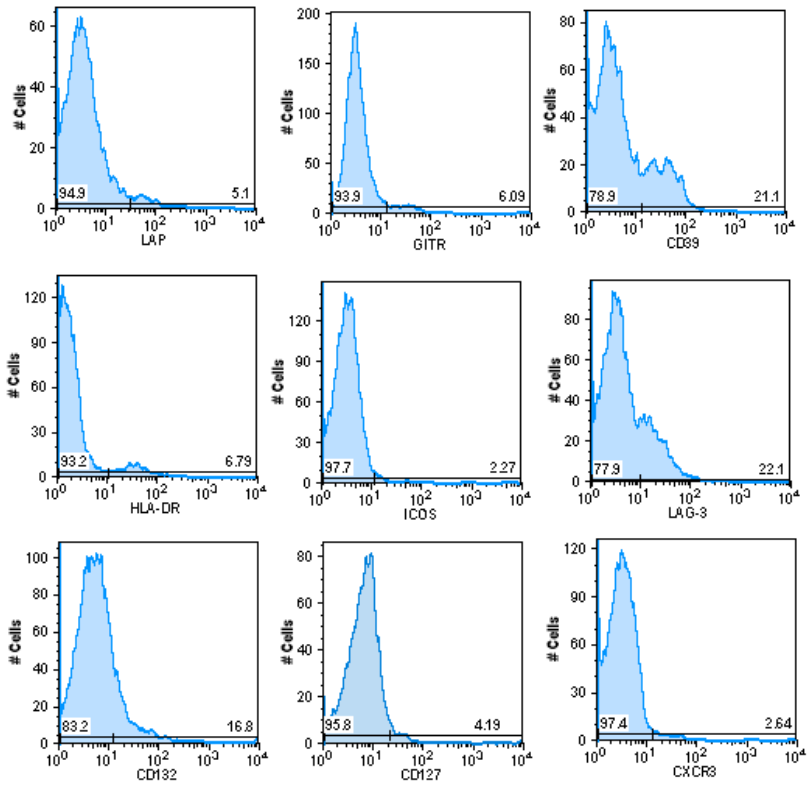


Figure 3.4 Representative characterization of CB Tregs (n=8). Histograms showing expression of different markers gated from $CD4^{pos}$ $FoxP3^{pos}$ population from CB. Results are shown as percentage of expression.

3.3 Discussion.

It has been shown, that Tregs pool, suffer phenotypic changes in an age-dependent fashion. Early in age, Tregs start with a naïve phenotype ($CD45RA^{pos}$) and undergo a preferential differentiation towards a central memory phenotype ($CD45RO^{pos}$) (Akbar *et al.*, 2007b; Seddiki *et al.*, 2006b). However, certain functional properties have been suggested to diminish in correlation to these phenotypic changes (Ayyoub et al., 2009; Haas et al., 2007). Nonetheless, Treg frequency is remarkably stable throughout life in humans (Santner-Nanan et al., 2008). It has been shown that Tregs pool is maintained throughout life due to rapidly dividing $CD4^{pos}$ $CD45RO^{pos}$ T cells in vivo (Vukmanovic-Stejic et al., 2006).

In agreement with what has been shown (Bresatz et al., 2007; Fritzsching et al., 2006; Godfrey et al., 2005; Santner-Nanan et al., 2008), I showed that CB Tregs represented 5 to 10% from overall $CD4^{pos}$ T cells. No statistical difference was depicted when compared to adult Tregs (6 to 10% from $CD4^{pos}$ T cells). Furthermore, CB Tregs were mainly $CD45RA^{pos}$ (~89%) compared to adult (18%). In addition, CB $FoxP3^{pos}$ $CD45RA^{pos}$ co-express CD31 (~80%), which has been shown to be a marker that together with CD45RA, specifically depicts cells that are RTE conserving a wide TCR repertoire (Haas et al., 2007; Junge et al., 2007). This has important considerations for their application in transplantation, since $CD45RA^{pos}$ $CD31^{pos}$ Tregs TCR properties are more likely to resemble and counteract the high frequency (~7%) of allo-reactive naïve effector T cells that mount the strong response against the allograft (Suchin et al., 2001). In addition, herein is shown that CB Tregs are mostly rTregs ($CD25^{pos}$

CD45RA^{pos} FoxP3^{low}), which have been shown to be *bona fide* Tregs *in vitro* and *in vivo* (Hoffmann *et al.*, 2006b; Miyara *et al.*, 2009; Seddiki *et al.*, 2006b). Furthermore, rTregs are highly proliferative and apparently have a major role in overall Treg pool homeostasis.

Additionally, CB Tregs constitutively express a repertoire of markers that has been shown to be important for their functionality. CB Tregs highly expressed CCR7 (~72%). It has been described that CCR7 expression on Tregs is paramount for their migration to lymph nodes and their allocation within the T-cell zone (Forster et al., 2008; Zhang et al., 2009). This particular process has been of great importance for Tregs suppression *in vivo* (Zhang et al., 2009). Furthermore, I also showed that CB Tregs co-express the lymphocyte adhesion molecule CD62L (~57%). In accordance, it has been demonstrated in a mice GvHD model, that only CD62L^{high} Tregs interfere with the activation and expansion of GvHD effector T cells within the secondary lymph nodes (Taylor et al., 2004).

In summary, CB has a higher frequency of the most homogenous population (CD45RA^{pos}) described so far of nTregs compared to adult PBMCs. Furthermore, they constitutively express homing receptors (CCR7 and CD62L) that will allow them to migrate *in vivo* to secondary lymph nodes. Lastly, most CB Tregs conserve the beneficial cellular properties of RTE T cells. As mentioned, this has major benefits for their application *in vivo*.

CHAPTER 4

OPTIMIZED METHODOLOGY TO SPECIFICALLY ISOLATE $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ POPULATIONS FROM CB USING A SINGLE STEP PROTOCOL

4.1 Introduction

There is currently a lack of specific markers in order to characterize Tregs properly. Nevertheless, FoxP3 is the most accepted marker for their characterization in steady state of non-inflammation ex-vivo and in-vivo (Fontenot *et al.*, 2005b; Seddiki *et al.*, 2006a). Since FoxP3 is an intracellular marker it cannot be used for isolation of viable Tregs. Thus, CD4 and CD25 have been used so far for Tregs isolation (Baecher-Allan, 2006; Hoffmann et al., 2006a; Sagoo et al., 2008). The isolated populations have been demonstrated to be suppressive in models *in vitro* and in *in vivo* (Sakaguchi et al., 2008; Shevach, 2009). Later on, it was demonstrated that $FoxP3^{pos}$ cells are mainly $CD127^{neg/low}$ (IL-7 α receptor) whereas effector cells are $CD127^{high}$ (Liu *et al.*, 2006; Seddiki *et al.*, 2006a). Depletion of $CD127^{high}$ cells from the isolated $CD25^{pos}$ fraction has been shown to improve substantially the purity of Tregs isolated from PBMCs (Hoffmann et al., 2009; Peters et al., 2008; Putnam et al., 2009). Most of the studies on Tregs isolation methods for clinical purposes have recommended multiple step protocols, mostly CD8, CD19 and most recently CD49d depletion (Kleinewietfeld et al., 2009; Peters et al., 2008; Riley et al., 2009). Nevertheless, low purity and yield continue to be an issue for further characterizations and stepping into bigger scale models and clinical trials. Latest

studies have shown that CD45RA^{pos} Tregs represent the most homogenous population among the Tregs pool (Hoffmann et al., 2006b). Unfortunately, less than 30% of adult Tregs are CD45RA^{pos}, resulting in a higher proportion of memory phenotype Tregs (CD45RO^{pos}) (Santner-Nanan et al., 2008; Vukmanovic-Stejic et al., 2006). In contrast, cord blood Tregs are mainly naïve CD45RA^{pos} (>80%) and have also been shown to be highly suppressive (Godfrey et al., 2005; Porter et al., 2006; Wing et al., 2005). Interestingly, cord blood CD4^{pos} CD25^{pos} population is a well-defined population with a clearer gap between its counterpart negative fraction (CD4^{pos} CD25^{neg}) (Figure 4.1B). In addition, they express the same level of FoxP3 as CD4^{pos} CD25^{high}cells from PBMCs (Figure 4.1C,D). Moreover, the level of CD25 expression among the CD4^{neg} population is barely evident (Figure 4.1B). Taking all this into consideration, cord blood makes a *bona fide* source for the isolation of Tregs using a single step CD25 isolation protocol.

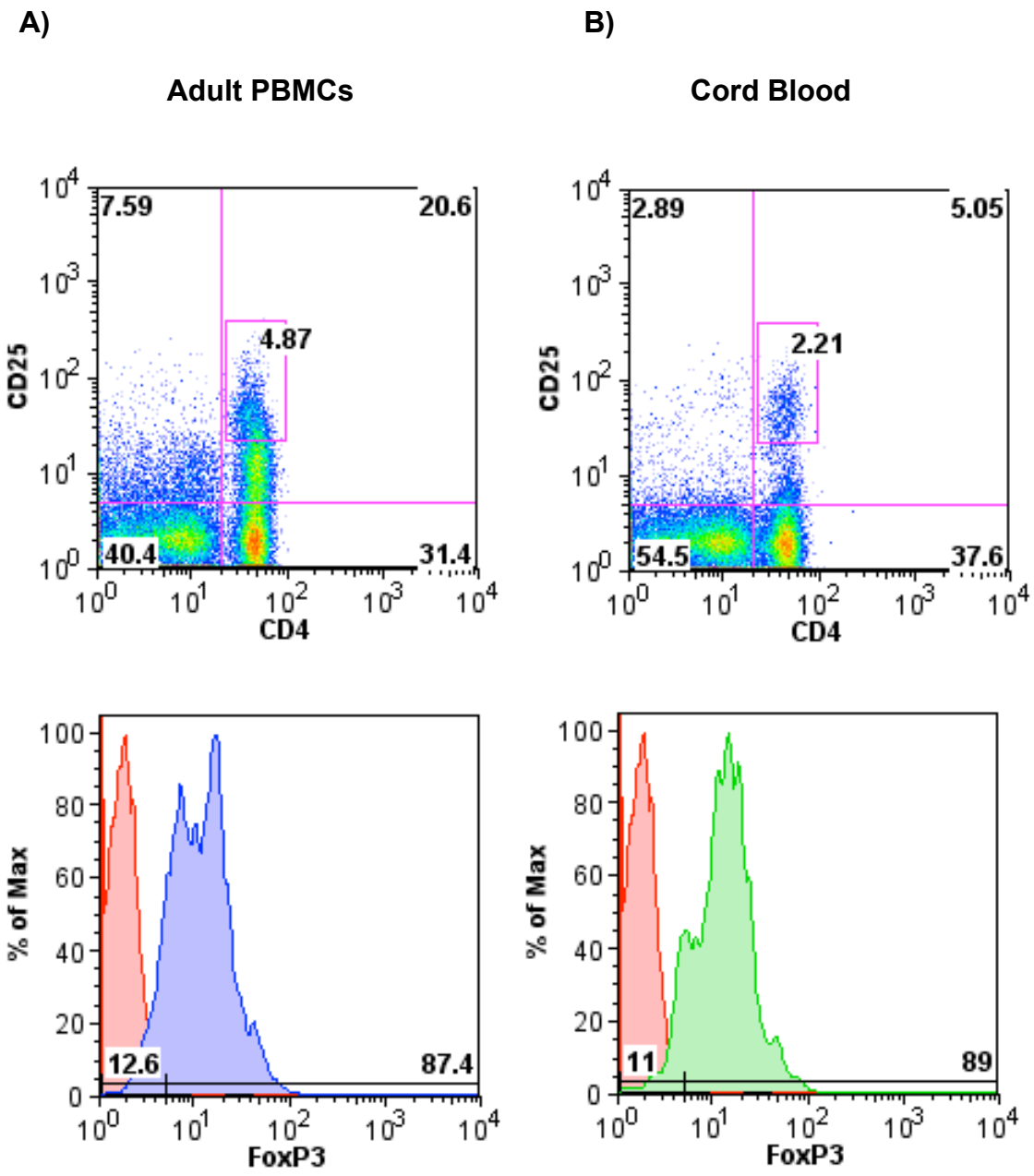


Figure 4.1 A representative characterization of CD4^{pos} CD25^{pos} populations in PBMCs and CB (n=10). This figure shows CD4 vs CD25 expression on **A)** adult PBMCs and **B)** CBMCs, gated on live lymphocyte gate. Histograms below show the level of FoxP3 expression of CD4^{pos} CD25^{high} cells from PBMCs (in **blue**) and CB CD4^{pos} CD25^{pos} cells (in **green**). Isotype control is depicted red.

4.2 Results

This chapter describes the optimization of the isolation of highly purified $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ T cells from cord blood using a single step protocol.

4.2.1 Cell density modifications for specific Tregs selection

Some groups have shown an increase in Tregs purity using lower concentration of CD25 microbeads with the intention to only capture the cells with the highest CD25 expression ($CD25^{high}$) (Baecher-Allan, 2006). This study tested also the effect of modifying the cell density before CD25 microbeads staining. The staining conditions described by the manufacturer protocol (Miltenyi Biotec, Germany) will be mentioned throughout this chapter as "standard protocol". Briefly, cells were resuspended in isolation buffer at 90μl per 10^7cells and stained with 10μl of anti-CD25 microbeads per 10^7cells leading to a cell concentration of ~$1.1x10^8$cells/ml (standard protocol). In parallel, CD25 isolation was also done using three different cell densities with their corresponding anti-CD25 microbeads concentration (1/9): A) ~$2.5x10^8$cells/ml; B) ~$3.3x10^8$cells/ml and C) ~$5x10^8$cells/ml. After staining for 15 mins in the dark at 4°C, each cell suspension was washed and resuspended in 500μl of isolation buffer (described in section 2.6) and passed through two LS columns as suggested by the manufacturer (Miltenyi Biotec, Germany). In order to reduce sample bias, the initial isolations were carried out from the same CB unit and repeated three times in different CB units.

4.2.2 Purity for $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ and level of contaminating $CD4^{pos}$ $CD127^{high}$ cells.

As mentioned in section 2.1 and in reference to previous studies (Liu *et al.*, 2006; Seddiki *et al.*, 2006a), Tregs were characterized as $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ T cells and effectors as $CD4^{pos}$ $CD127^{high}$ T cells. The latter can also be sub-divided in function of CD25 expression as shown in figure 4.2 but for statistical analysis effector cells were defined as $CD4^{pos}$ $CD127^{high}$ without CD25 subdivision. This study shows that high purity for $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ T cells can be achieved using a single step method (CD25 positive selection) and that cell density during anti-CD25 microbeads staining was found crucial factor for these results. Figure 4.2 shows purity of CD25 positive isolations using the same CB sample. These results show that a cell density of $>2x10^8$ cells/ml dramatically increases the purity of Tregs and therefore decreases the level of contaminating effector cells. Isolations using densities between ~$3.3x10^8$cells/ml and ~$5x10^8$cells/ml showed the highest purities for Tregs compared to the standard protocol (mean 89% and 90% vs 79%, respectively, n=3 p=0.001). This study did not show any statistical difference between ~$3.3x10^8$cells/ml and ~$5x10^8$cells/ml cell densities. Therefore, in order to have an easier translation to larger scale isolation, further isolations were carried out using a cell density of ~$3x10^8$cells/ml and will be mentioned in this study as “new method”.

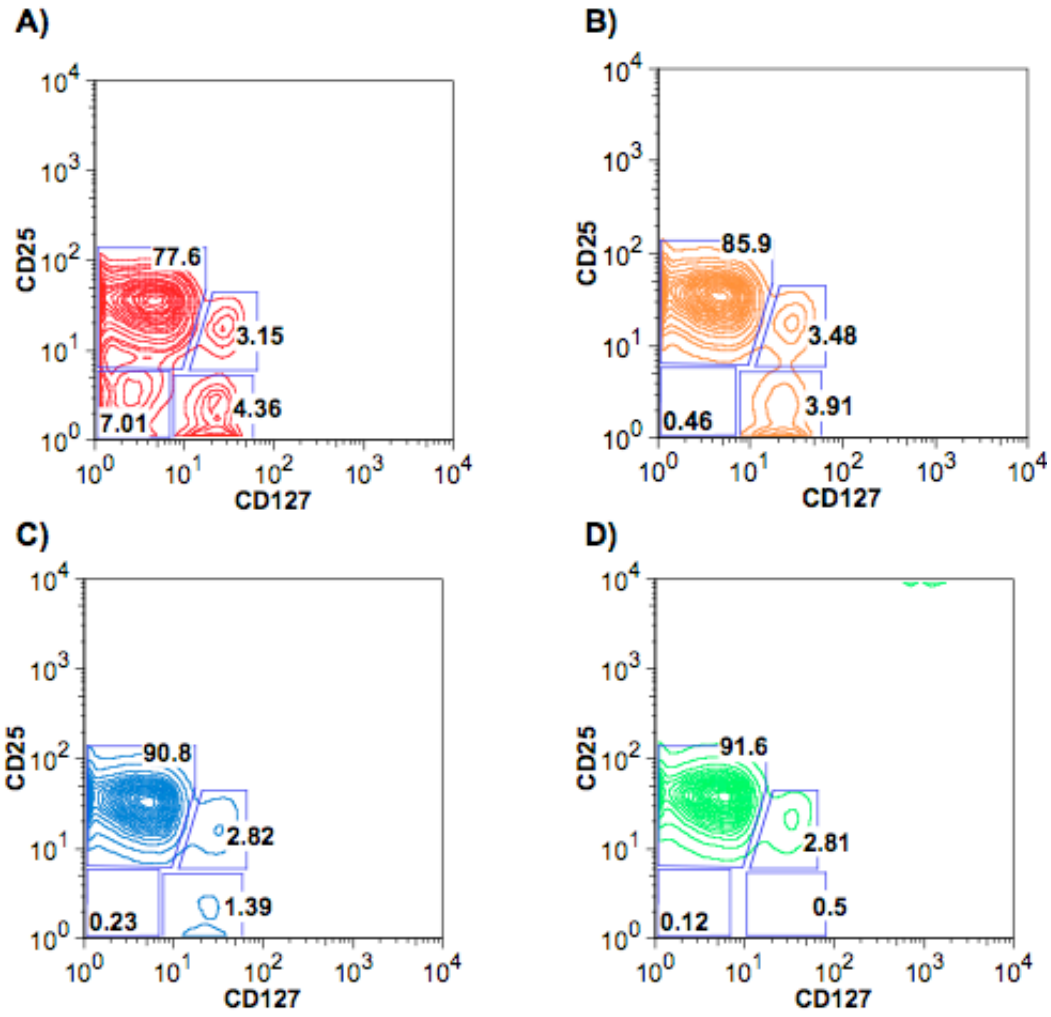


Figure 4.2 Flow cytometry analysis of CD25 positive isolations from the same CB unit using different cell densities (representative data, n=3). Contour plots gated on $CD4^{pos}$ cells, plotted against CD25 and CD127. Tregs are gated as $CD25^{pos}$ $CD127^{low/neg}$, contaminating cells are subdivided in three sub-populations: $CD25^{neg}$ $CD127^{neg}$, $CD25^{neg}$ $CD127^{high}$ and $CD25^{pos}$ $CD127^{high}$. Numbers represent percentages of each sub-population. **A)** Cells were stained at ~$1.1x10^8$cells/ml (standard protocol), **B)** at ~$2.5x10^8$cells/ml, **C)** at ~$3.3x10^8$cells/ml and in **D)** at ~$5x10^8$cells/ml.

4.2.3 "New method" vs. standard protocol for large-scale Tregs isolation.

In order to test if these modifications can be translated into larger scale isolation, complete CB units (a range between 1.8 to 2.2x10^8 CBMNCs) were used for CD25 positive isolation using either the standard protocol (~1.1x10^8cells/ml) or the new method (~3x10^8cells/ml). As shown in Figure 4.3, a higher proportion of Tregs ($CD4^{pos}$ $CD25^{high}$ $CD127^{low}$) was observed with the new method compared to the standard protocol (mean 89% of the $CD4^{pos}$ cells, range: 86-94% vs mean 72% range 54-89% respectively, p=0.03). Also the proportion of contaminating effector cells, was notably reduced by using this new method ($CD4^{pos}$ $CD127^{high}$) (mean of 6% range: 3.6 to 9% vs mean 23% range: 7.3 to 38%, p= 0.01). Although not significant, a noticeable difference was seen in the proportion of $CD4^{pos}$ cells (mean 86% vs 80% respectively, p=0.6) and/or the proportion of $CD4^{pos}$ $CD25^{pos}$ cells (mean 86% vs 76% respectively, p=0.1).

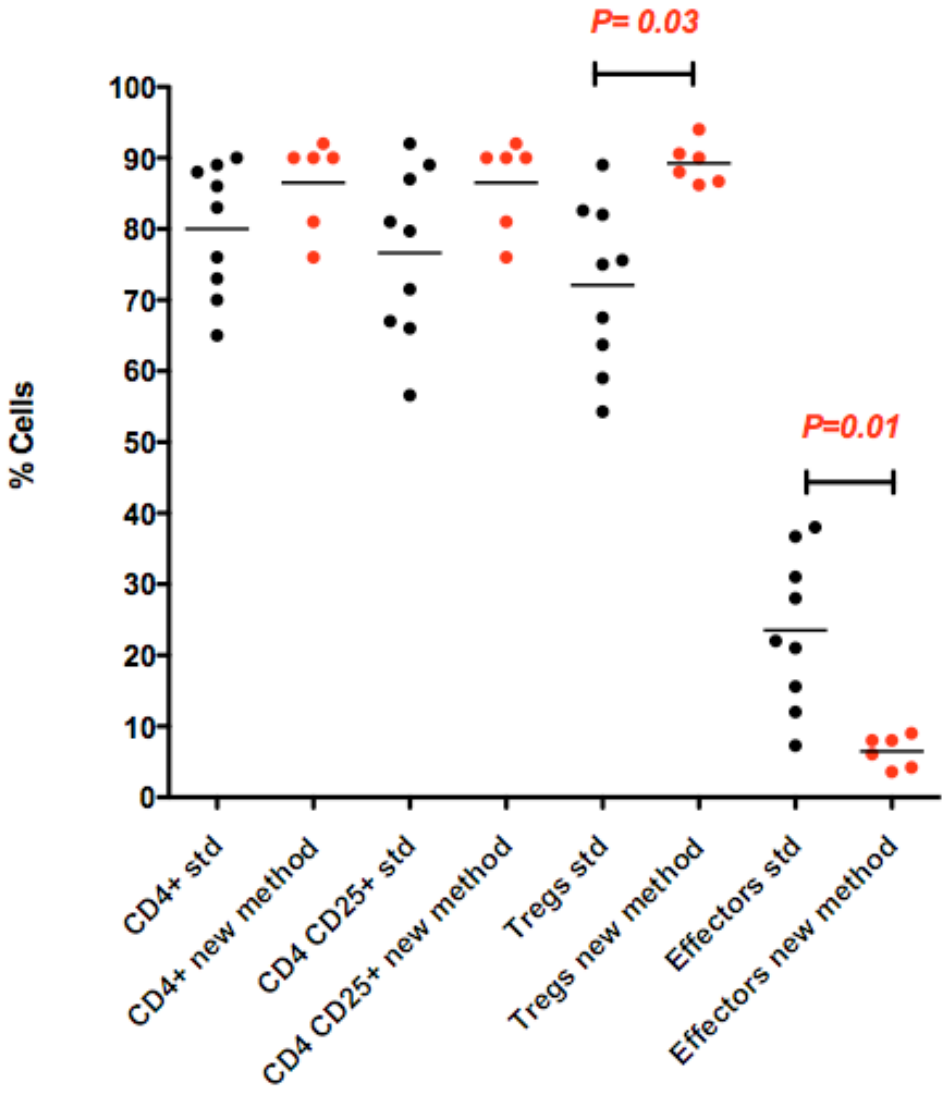


Figure 4.3 Percentage of different subpopulations using the two methods of isolations. Standard (std, black dots n=9) and the new method (**red dots n=6**). The percentage of $CD4^{pos}$ was taken from the lymphocyte gate. The rest of the subpopulation percentages were taken from $CD4^{pos}$ cells. $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ was defined as Tregs and $CD4^{pos}$ $CD127^{high}$ as effectors. Mean values are shown of each subpopulation. Significance difference (p=<0.05) was done by Students t test.

4.2.4 Yield of isolated Tregs

The final yield of Tregs was calculated as the percentage of $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ from the initial cell count used for the isolation. The new method resulted in a much higher yield of Tregs compared to the standard protocol (mean 0.4%, range: 0.3 to 0.6%, (n=6) vs mean 0.2%, range: 0.1 to 0.4%, (n=9), respectively, *p*=0.007) (Figure 4.4).

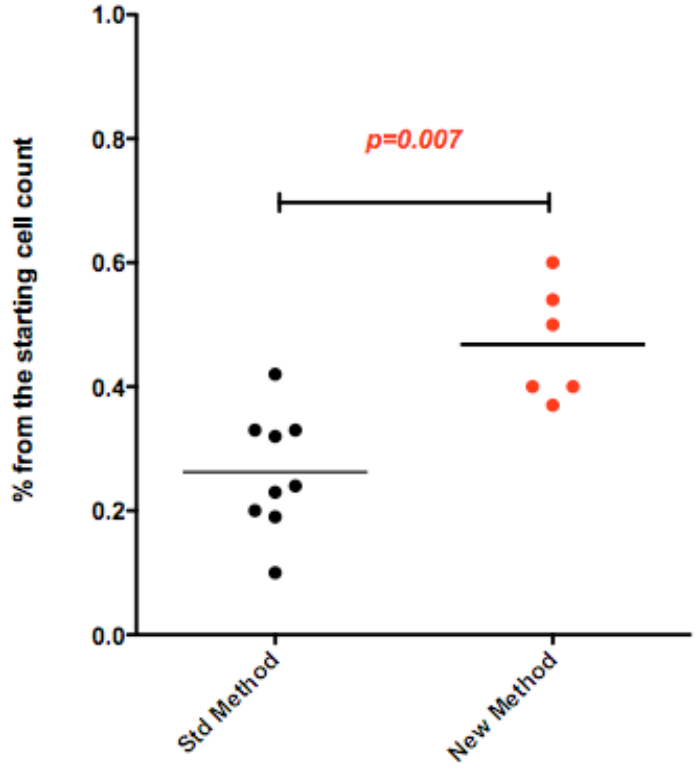


Figure 4.4 Final yields of Tregs determined from the initial cell count. Shown is the mean proportion of defined Tregs in each method. The standard method is depicted with black dots (n=9) and the new method in red dots (n=6). Student *t* test was done to evaluate the difference between groups.

4.2.5 Selected Tregs suppressive ability

Since the main functional characteristic of Tregs is their suppressive ability, we tested the isolated populations in functional assays. Both methodologies generated populations that demonstrated suppression to allo-stimuli (p= <0.04). Overall the new method did not show a significant difference with the standard protocol regarding percentage of suppression (median 96% at 1:2 ratio (Tregs/effectors) vs 70%, p=0.1 and at 1:5 ratio of 60% vs 68%, respectively (n=4, p=0.7) (Figure 4.5).

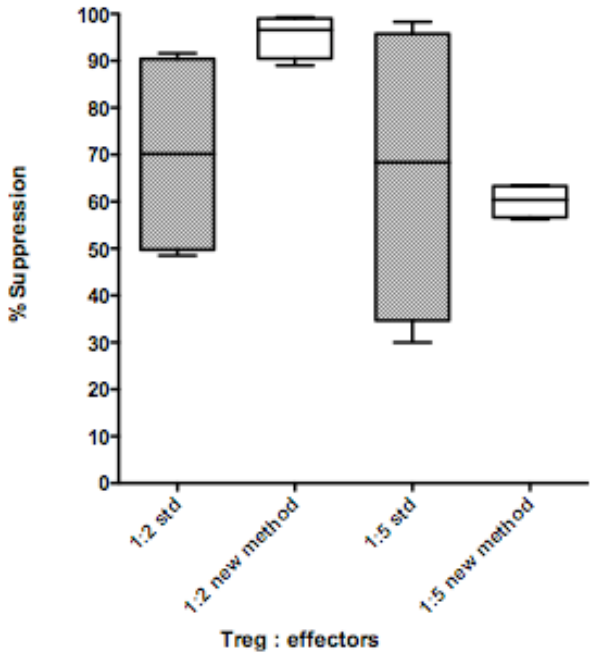


Figure 4.5 Suppressive ability of cell subsets isolated with both protocols. Percentage of suppression at different Tregs:effector ratios using CD25 positive isolations from both protocols tested in the same MLCs (n=4). Box and whisker plots are shown.

However, the standard protocol cohort had a coefficient of variation >31% (at 1:2 or 1:5 Treg/effectors ratios) compared to the new method cohort which had a coefficient of <6%. To further elucidate if this was because of variable purity among isolations we conducted a Pearson test to evaluate correlation between purity and suppression (Figure 4.6). A good correlation was found between the proportion of $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ cells within isolations and percentage of suppression (r^2=0.57; *p*=0.02) (Figure 4.6 A). In contrast, this was not observed when gated on $CD4^{pos}$ $CD25^{pos}$ cells (r^2=0.26; *p*=0.5)(Figure 4.6 C). Consistent with the correlation of $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ cells with suppression, $CD4^{pos}$ $CD127^{high}$ effector cells showed an inverse correlation with suppression (r^2=0.64; *p*=0.01)(Figure 4.6 B).

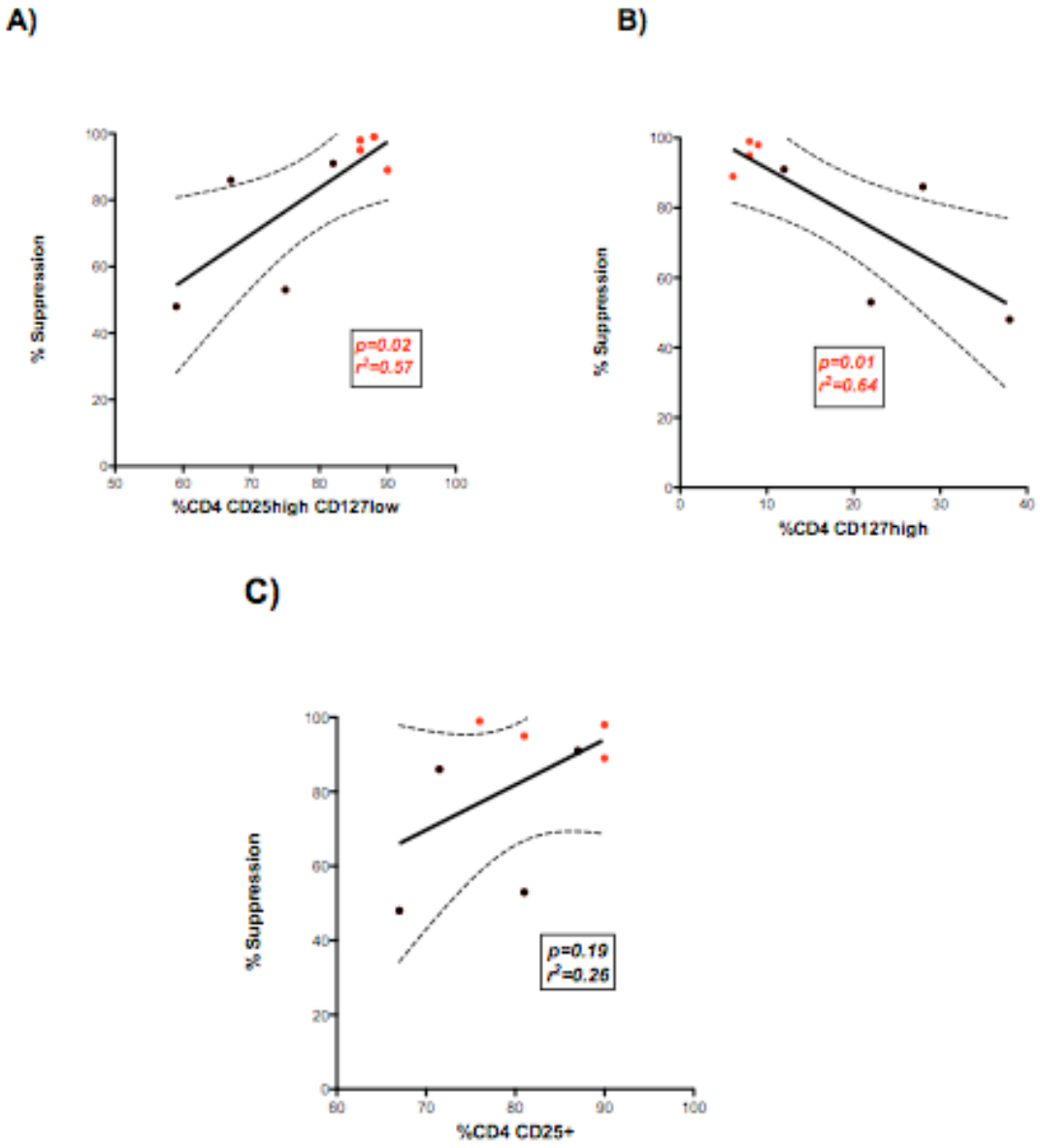


Figure 4.6 Correlation between $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ (A), $CD4^{pos}$ $CD127^{low}$ (B) and $CD4^{pos}$ $CD25^{pos}$ (C) percentages with suppression in MLC at 1/2 ratio (Tregs/effectors). Red dots represent isolations using the new method (n=4) and **black** dots using the standard protocol (n=4). Mean values and 95% CI are shown. Analysis was done using a Pearson test and two-tailed *t* test.

4.3 Discussion

Several groups have described CB as a rich source of well-defined $CD4^{pos}$ $CD25^{pos}$ cells, which are mainly naïve Tregs (Chang et al., 2005; Godfrey et al., 2005; Hippen et al., 2008; Porter et al., 2006). The lower proportion of effector cells ($CD4^{pos}$ $CD127^{high}$) among the $CD25^{pos}$ as well as the scarce $CD25^{pos}$ cells among the CD4 negative fraction makes it an optimal source for a one step Ab clinical grade selection.

FACs sorted isolations gating on the $CD4^{pos}$ $CD25^{high}$ have shown high levels of purity ~98% but at the expense of low yields (<0.1%) (Baecher-Allan, 2006). Methods using microbeads conjugated Ab to CD25 have higher yields (0.3 to 0.4%) but with purities that range from 72 to 92% gating on $CD4^{pos}$ $CD25^{pos}$ (Hoffmann et al., 2006a; Kleinewietfeld et al., 2009; Peters et al., 2008; Wichlan et al., 2006). However, assessing the purity by these criteria can be misleading especially with adult PBMCs, as there can be a substantial number of effector cells ($CD4^{pos}$ $CD127^{high}$) among the $CD25^{pos}$ fractions. Employing CD25 vs CD127 gate on $CD4^{pos}$ cells is a more accurate Tregs characterization since it has been shown to have a strong correlation with FoxP3 ex-vivo and in-vivo in steady state (Liu *et al.*, 2006; Seddiki *et al.*, 2006a). These results were also confirmed phenotypically and functionally. This study describes a protocol for Tregs isolation from CB, which has led consistently to high $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ cell purity (mean 89%) using a single step CD25 selection. Additionally, this purity was achieved without a drop in yield. Furthermore, the yield was doubled (mean 0.4%) compared to the standard protocol (mean 0.2%) and even greater to FACs sorted isolation reported by others (0.1%) (Baecher-

Allan, 2006). Another factor of great importance regarding Tregs isolation methods is the percentage of contaminating effector cells ($CD4^{pos}$ $CD127^{high}$). This new method efficiently depletes this population to <9%.

All CD25 positive isolations were suppressive when tested in functional assays. Nevertheless, the variability seen within the standard protocol isolations among the percentage of suppression was important. This effect was strongly correlated with the variability in the proportion of $CD4^{pos}$ $CD25^{high}$ $CD127^{low}$ cells. In concordance we observed an inverse correlation with suppression and the proportion of contaminating effector cells ($CD4^{pos}$ $CD127^{high}$). In agreement with other groups (Liu *et al.*, 2006; Seddiki *et al.*, 2006a), gating on $CD4^{pos}$ $CD25^{pos}$ cells does not clearly characterize Tregs as we have shown no suppressive correlation when employed this gating strategy.

In summary we found that increasing the cell density >3 fold from the standard staining protocol in conjunction with suboptimal doses of anti-CD25 microbeads dramatically improves the purity and yield resulting in highly suppressive populations. These findings also suggests that a threshold of contaminating effector cells (>10% of $CD4^{pos}$ $CD127^{high}$), within the cells isolated, may jeopardize the suppressive properties of Tregs. However, the inevitable level of contaminating $CD4^{pos}$ $CD25^{pos}$ $CD127^{high}$ effector cells (<10%), may also be required for Tregs activation and metabolic fitness (Shevach, 2009; Vignali et al., 2008).

CHAPTER 5
SUPPRESSIVE FUNCTION OF INDIVIDUAL AND POOLED CB TREGS (pCB TREGS)

5.1 Introduction

It has been clearly demonstrated that Tregs from the host as wells as from the donor potently suppress allo-immune responses *in vitro* and *in vivo* (Edinger et al., 2003; Joffre et al., 2008; Taylor et al., 2004; Trenado et al., 2003; Tsang et al., 2009). However, in order to achieve an effective suppression *in vivo*, most of the experiments used host Tregs in a 1:1 and even 3:1 Treg:effectors cell ratios (Nomura et al., 2006; Sagoo et al., 2008; Taylor et al., 2004).

Interestingly, it has also been shown that naïve Tregs from a third party donor can be used to prevent GvHD and helps bone marrow engraftment (Hippen et al., 2008; Steiner et al., 2006). Therefore, in the context of direct allorecognition, naïve Tregs are able to cross-react upon alloantigens, similarly as their counterpart conventional effector T cells. In accordance, a wide TCR repertoire overlap has been demonstrated between Tregs and their counterpart subset $CD4^{pos}$ $CD25^{neg}$ cells (Fazilleau et al., 2007; Hsieh et al., 2006; Pacholczyk et al., 2006; Vukmanovic-Stejic et al., 2006). Thus, if ~7% of un-manipulated naïve T cells are able to respond to allo-stimuli (Suchin et al., 2001), likewise, naïve Tregs could be expected to react upon encounter to allo-antigens *in vivo* (Nomura et al., 2006). However, in spite of having nTregs that are able to suppress allo-responses, most of the studies emphasize the necessity to also outcompete effector T cells with at least an equivalent number of Tregs

(Hoffmann et al., 2006a; Riley et al., 2009; Sagoo et al., 2008) hence, skewing the balance towards a regulatory milieu is also of great importance.

Tregs only constitute between 4 to 10% of the total $CD4^{pos}$ T cells in peripheral blood (Baecher-Allan et al., 2001; Santner-Nanan et al., 2008). Thus, the requirement to establish expansion protocols have been the only solution so far to achieve optimal cell numbers for Treg immunotherapy. Current expansion protocols either using polyclonal or antigen specific stimuli have shown encouraging results, with approximately >200 fold expansion in 2 to 3 weeks culture (Battaglia et al., 2006; Hoffmann et al., 2004; Peters et al., 2008; Putnam et al., 2009; Trenado et al., 2006). However, after 2 weeks of culture most of these protocols have shown down-regulation of FoxP3 and an important level of conversion or outgrowth of Th-17, Th-2 or Th-1 effector cells (Hoffmann et al., 2009; Hoffmann et al., 2006b; Putnam et al., 2009). Infusion of effector T cells that may cause unwanted damages to the host or the graft is the main concern regarding Tregs immunotherapy, thus, emphasis has been allocated to this matter (Hoffmann et al., 2006a; Riley et al., 2009).

5.2 Results

The objectives of this chapter are firstly, to test the “third party” approach using CB Tregs to suppress alloresponses. Secondly, in order to acquire optimal cell numbers in a more practical approach, the modality of pooling multiple mismatch CB Tregs will be explored, hereafter called **pCB** Tregs. This approach not only would help to achieve optimal numbers for immunotherapy when requested, but also pooling naïve CB Tregs at their steady state prior activation will maintain

their wide TCR repertoire, cellular proliferative properties and will therefore increase the possibilities of an efficient suppression *in vivo*. In addition, since the number of cells is as important as their specificity, and in the context of acquiring the optimal cells and number for immunotherapy, I would also test the option of using pCB Tregs as an adjuvant to host Tregs. At last, since pooling CB units would imply more expression of alloantigens, I will also test their immunogenicity.

5.2.1 CB Tregs effectively suppress alloresponses.

As described in section 2.5.1, one-way MLCs were done to measure the capacity of Tregs to suppress alloresponses *in vitro*. This assay is intended to measure specifically the ability of Tregs to block direct allorecognition responses. A total of 8 MLCs were done, Tregs either from the host or CB were added at different Tregs:effectors cells ratios (1:2, 1:5 and 1:10). All MLCs developed stimulation index >7. As shown on section 2.3 and 3-2.3, all Tregs isolated used for functional assays had purities >89% for $CD25^{pos}$ $CD127^{low}$ (range 89 to 96%).

CB Tregs were effectively suppressive at all ratios. Although a tendency of better suppression can be seen when host Tregs are used, on overall there was no significant difference between host and CB Tregs suppression. (Figure 5.1)

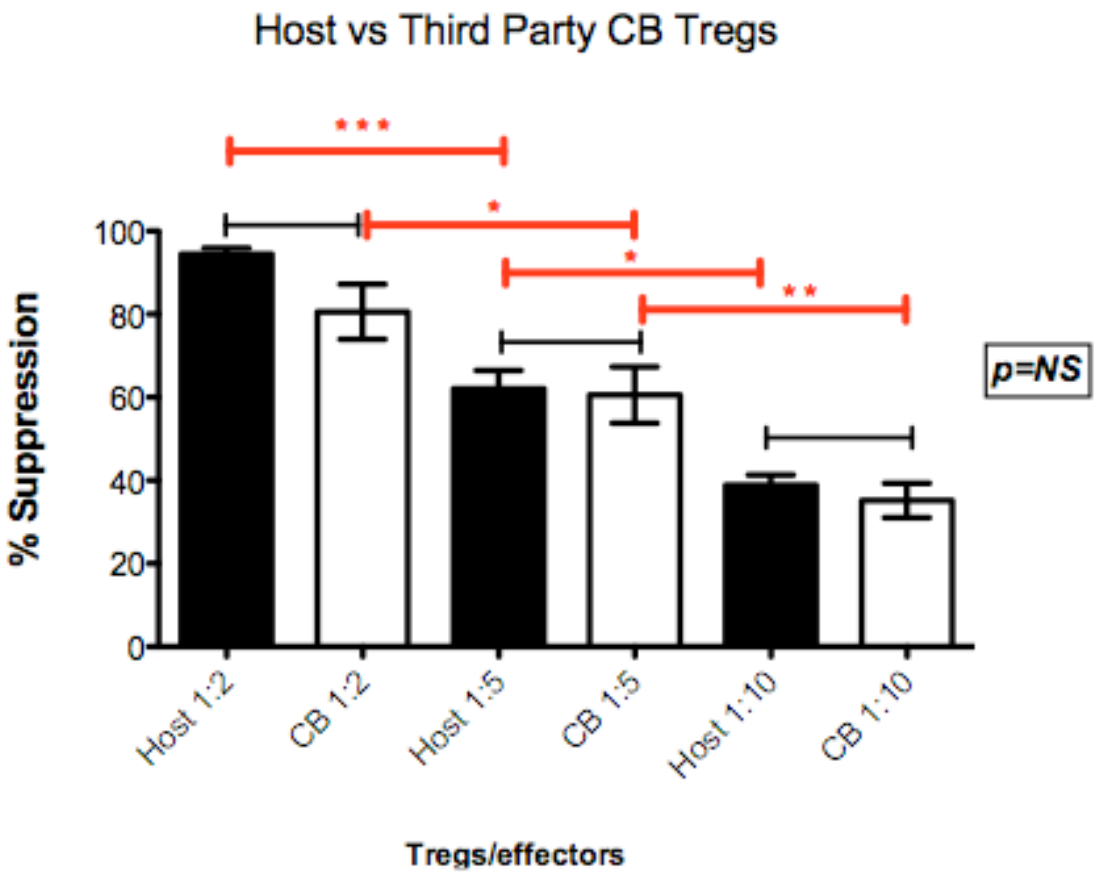


Figure 5.1 Summary of suppression assays using host and CB Tregs. MLCs (n=8) were done with the addition of either Tregs from the Host (**black bars**) or CB (white bars) at different Tregs:effectors ratios (1:2,1:5 and 1:10). Results are shown as percentage of suppression, Mean ± SEM. **Red lines** shows the differences with statistical significance (* ***p***=0.05, ** ***p***=0.003 and *** ***p***=<0.001) and **black lines** the ones with no statistical significance (**NS**). A One-way ANOVA with Tukey's post-test was done for statistical analysis.

5.2.2 pCB Tregs effectively suppress MLC without loosing their individual suppressive capacity.

In order to establish if CB Tregs could be pooled without loosing their individual suppressive capacity, Tregs from 3 mismatch CB units were isolated and added to MLCs (n=10). Individual CB Tregs or pCB Tregs were added at different Tregs:effector cell ratios (1:2,1:5 and 1:10). As shown before, individual CB Tregs were efficiently suppressive and in addition, pCB Tregs were constitutively suppressive at all ratios. Most importantly, when CB Tregs were pooled, their suppressive ability was maintained (Figure 5.2).

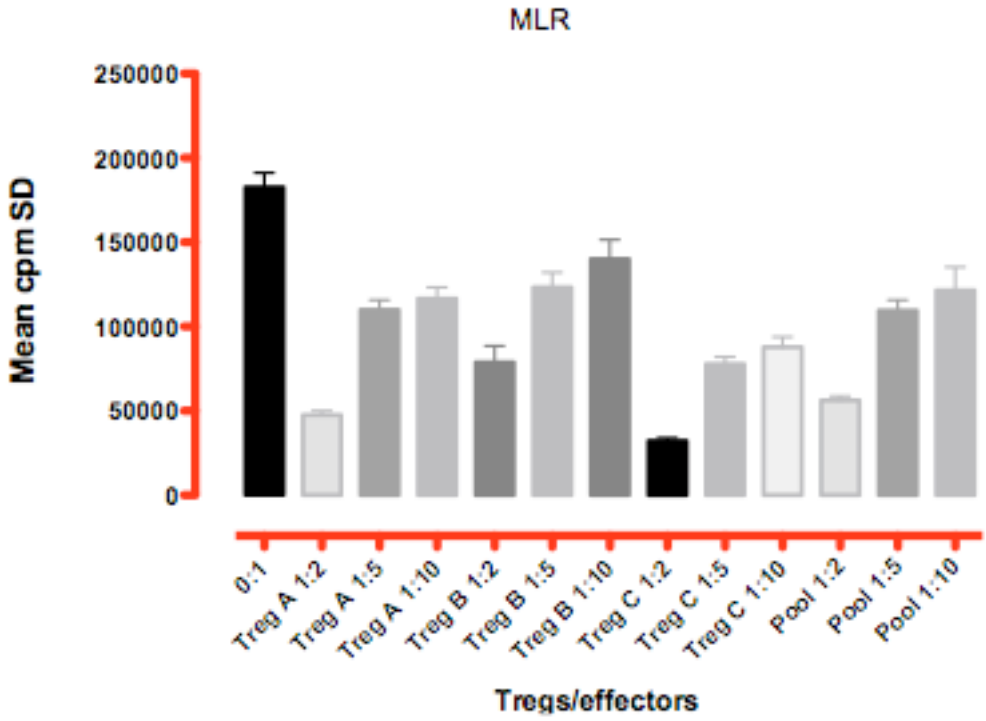


Figure 5.2 Representative suppression assay using individual vs pCB Tregs (*n*=10). Tregs isolated from 3 CB units (allocated as A, B and C) added either individually or as a pool (A+B+C) at different Tregs:effectors cell ratio (1:2,1:5 and 1:10). Results are shown as Mean c.p.m ± SD.

Interestingly, in 4 out of the 10 experiments, pCB Tregs showed better suppression (synergy pattern, Figure 5.3 **B**) compared to individual CB Tregs, whereas the rest of the experiments showed an average level of suppression (Figure 5.3 **A**).

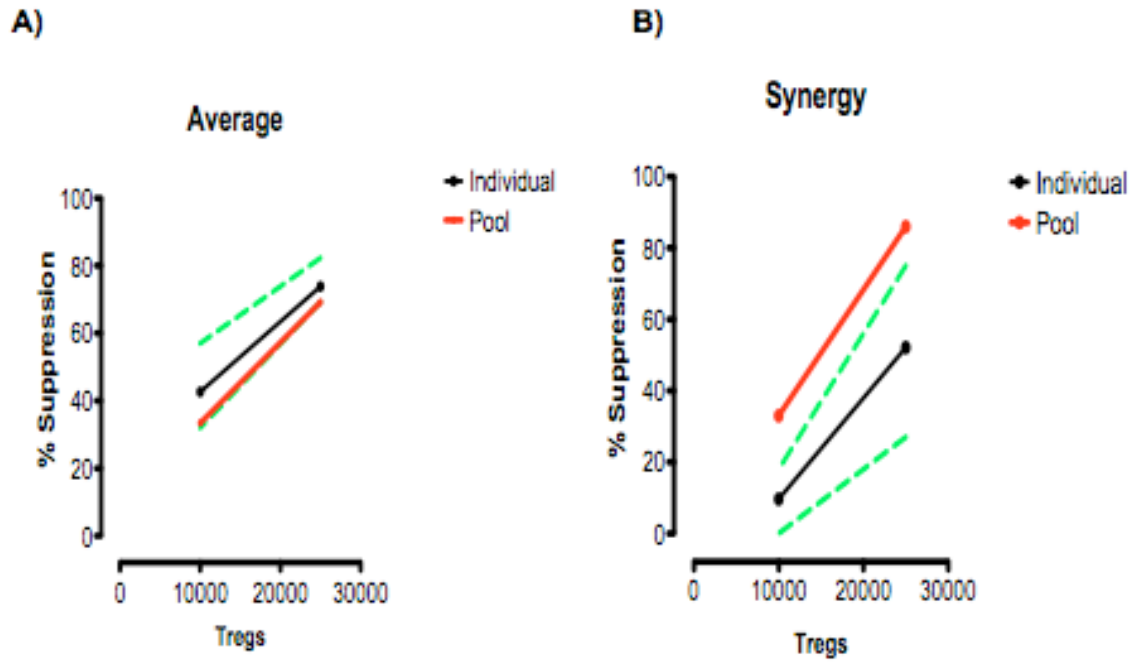


Figure 5.3 Suppression patterns in MLC using individual vs pCB Tregs (*n*=10). A) Representative graph of the experiments (60%) showing an average suppression using pCB Tregs. B) A representative experiment showing synergy suppression using pCB Tregs (40%). Results are shown as % of suppression. **Black** lines represent the mean value of suppression of the three individual CB Tregs and **green dotted** lines represent the 95% CI. **Red** lines represent the mean value of suppression using pCB Tregs.

In order to test further if "pooling" CB Tregs increases the cell frequency able to respond to alloantigens, CB Tregs were CFSE labeled and challenged either individually or pooled against a total of $1x10^5$ irradiated PBMCs for 6 days. Parallel suppression assays were done with CFSE labeled $CD4^{pos}$ $CD25^{neg}$ and non-stained CB Tregs in order to also test their suppressive function. As shown in Figure 5.4 **A**, pCB Tregs proliferated greater than individual CB Tregs when

challenge with allo-stimuli. Moreover, both individual or pCB Tregs require allo-stimuli for proper proliferation, since no proliferation was seen after 6 days without co-cultured with irradiated PBMCs. These results, suggest that pooling enables a wider repertoire for allorecognition. In addition, parallel suppression assays demonstrate CB Tregs and pCB Tregs suppressive efficiency (Figure 5.4 **B**).

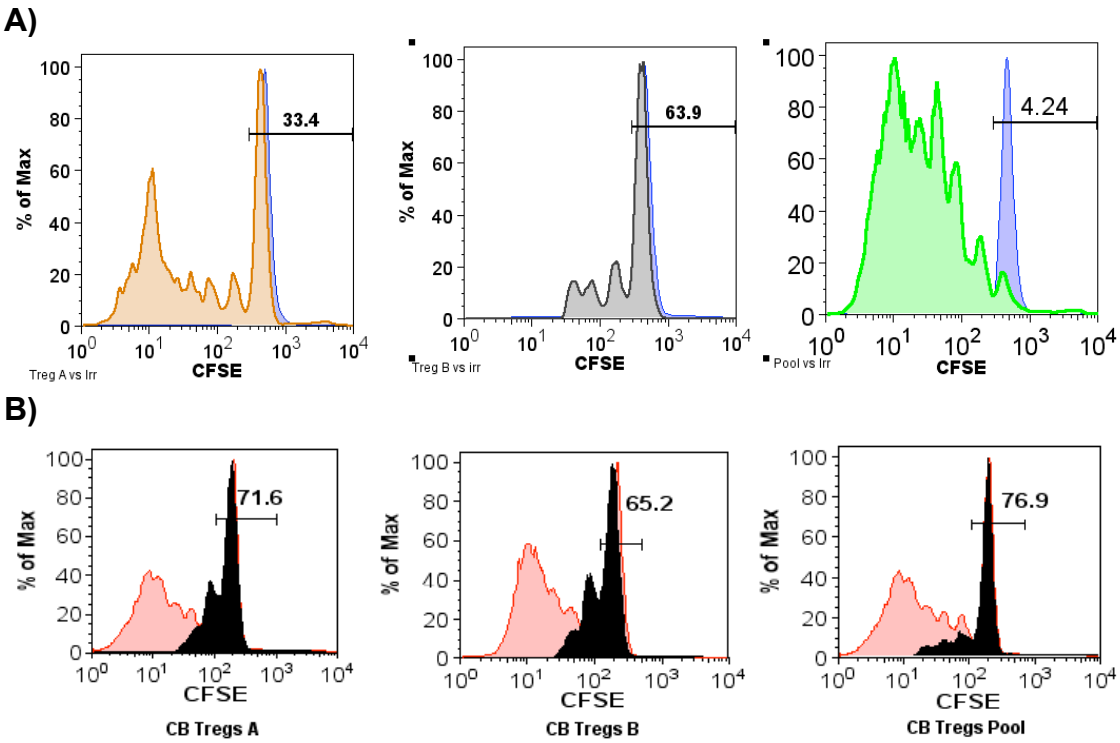


Figure 5.4 Representative histograms with CFSE labeled Tregs against allo-stimuli and parallel functional assay (n=3). A) Individual CFSE stained CB Tregs (**Tregs A, Tregs B**) stimulated either individually or **Pooled** with 1x10^5 irradiated PBMCs for 6 days. Blue histograms represents, the cell subset cultured without stimuli. B) Functional assay using CFSE stained $CD4^{pos}$ $CD25^{neg}$ subset stimulated with soluble CD3/CD28 Ab at 1/2.5μg/ml for 4 days. CB Tregs were added in a **1:2** Tregs effector cell ratio (**black histograms**). Red histograms represent, 0:1 Tregs: effector cell ratio. Results are shown as percentage of non-proliferating cells.

5.2.3 Pooling CB units prior CD25 isolation does not affect Tregs suppressive function.

As described in section 4.2.3 pooling of CB units, prior isolation is a feasible approach without affecting the purity post-isolation for $CD4^{pos}$ $CD25^{pos}$ $CD127^{low}$ cells. In order to complement these data and to test the suppressive function of the pCB Tregs, a series of $CD25^{pos}$ isolations were done from previously pooled CB units (range: 2 to 5 CB units) and used in MLCs at different Tregs:effectors cell ratio (1:2,1:5 and 1:10). Data was compared to all the suppression assays previously done using individual CB Tregs. In order to decrease variability, the same MLCs (*n*=15) were repeated using the same healthy controls used in the first experiments.

As shown in Figure 5.5, this approach did not affect the suppressive function of the pCB Tregs. In agreement with the previous results, no statistical difference regarding overall percentage of suppression was found between pCB Tregs and individual CB Tregs at all ratios.

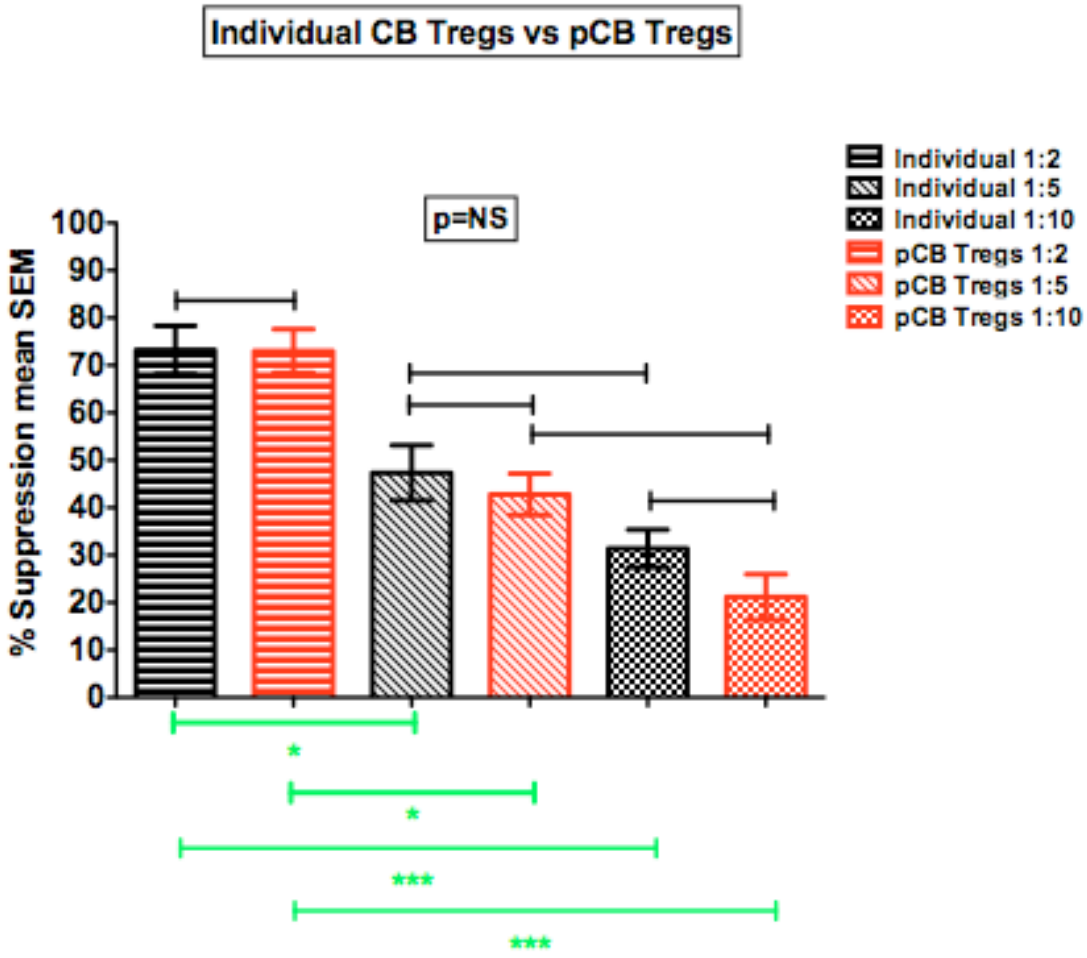


Figure 5.5 Suppression assays using previously pooled CB units Tregs vs individual CB Tregs (*n*=15). Results are shown as % of suppression ± SEM. pCB Tregs are depicted in **red** bars and individual CB Tregs in **black** bars at different Tregs:effector cell ratios. **Green** lines shows the difference among groups that have statistical difference (**p*=0.05, *** *p*=<0.001), whereas **black** lines shows the ones with no statistical difference **(NS)**.

5.2.4 pCB Tregs suppression is diminished in a non-APCs system.

As described in Chapter 1, we can summarize Treg mechanism of suppression whether they act directly on APC or effector cell subsets (Shevach, 2009). To further analyse in this context the mechanism of suppression of pCB Tregs, a non-APC system was done using a polyclonal stimuli. A total of $5x10^4$ $CD4^{pos}$ $CD25^{neg}$ cells were stimulated with soluble CD3/CD28 Ab (1/2.5μg/ml) and pCB Tregs were added at different Tregs:effector cells ratio (1:2 and 1:5). As shown in Figure 5-1.5 pCB Tregs showed a reduced level of suppression compared to the suppression observed on previous MLCs. This may suggest that the suppression of pCB Tregs is at least dependent on the presence of APCs. However, it is important to take into consideration that different stimuli were used in the two assays (MLCs and polyclonal), hence, different levels of response.

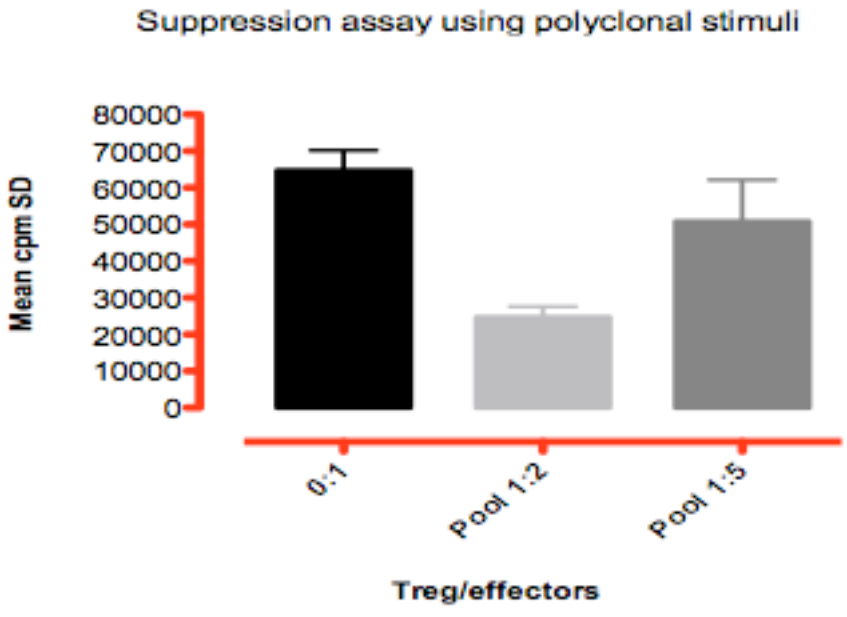


Figure 5.6 Suppression assay using polyclonal stimuli (representative of n=3). Adult $CD4^{pos}$ $CD25^{neg}$ were stimulated with soluble CD3/CD28 Ab at1/2.5μg/ml and co-cultured with pCB Tregs at 1:2 and1:5 Tregs:effector cells ratio for 4 days. Results are shown as Mean cpm ± SD.

It has been suggested that Tregs suppression can be underestimated when measured by H^3 thymidine uptake (Venken et al., 2007). Mainly because every proliferating cell, that is used in the assay will capture the radionuclide. In addition, since it has also been shown that Tregs can proliferate *in vitro* (Miyara et al., 2009), we cannot differentiate specifically the proliferation from each cell subset (Treg and effector T cells). To further test the suppression potency of pCB Tregs and to appreciate more the direct impact on effector T cells in the presence of pCB Tregs in a non-APC system, CFSE labeled $CD4^{pos}$ $CD25^{neg}$ T cells were stimulated with polyclonal stimulus (as previously described) with or without pCB Tregs (1:2 Tregs:effector cells ratio) for 4 days. Different activation markers as well as markers important for Tregs functions, were assessed from the CFSE+ population and correlated to their proliferative response.

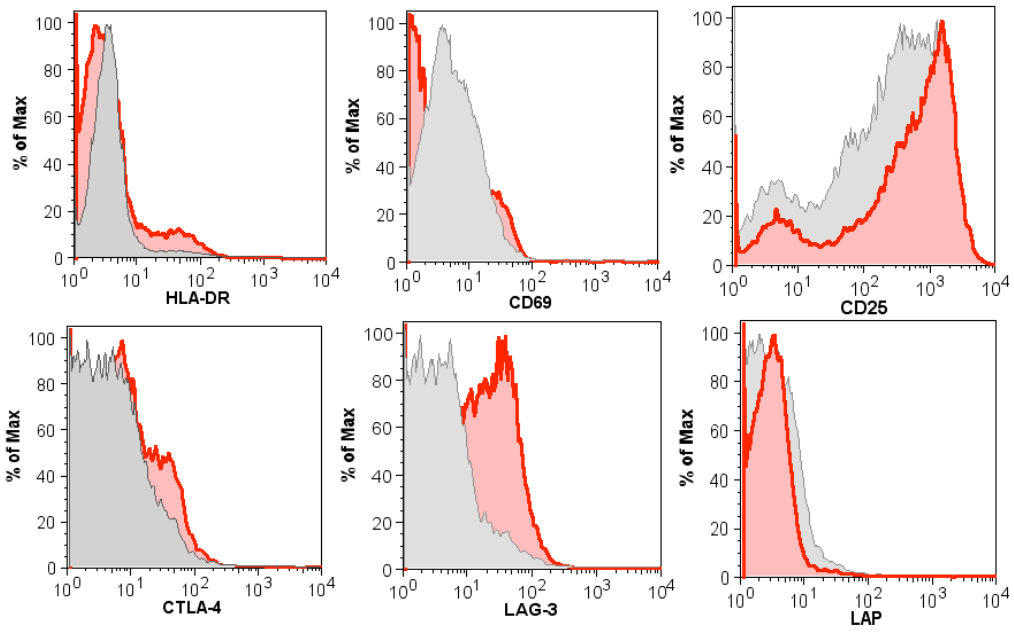
pCB Tregs showed 52% suppression in agreement with the level detected with H^3 Thymidine uptake (Figure 5.7 **B**). Even though less cells proliferated when co-cultured with pCB Tregs, effector T cells were able to up-regulate CD25, CD69 and HLA-DR (Figure 5.7 **A**). However, pCB Tregs potently inhibited effectors differentiation into cytokine secreting Th-1 cells, as they potently blocked IFN-γ secretion (Figure 5.7 **B**).

CTLA-4 and LAG-3 are normally up-regulated by activated T cells (Rudd et al., 2009; Triebel, 2003). No difference on the expression of theses markers was observed when effector T cells were co-cultured with pCB Tregs. Interestingly, a greater amount of effector T cells became LAP^{pos} when co-cultured with pCB Tregs, in spite of the reduced numbers of cells (Figure 5.7 **A**). These cells may

have been converted to iTregs, since it has been shown that iTregs can be developed from $CD4^{pos}$ $CD25^{neg}$ after activation in the presence of TGF-β (Chen et al., 2003). These cells constitutively up-regulate the latent associated peptide (LAP), which is the inactive form of TGF-β (Andersson et al., 2008).

Furthermore, pCB Tregs highly up-regulated CD39 expression (Figure 5.8). CD39 expression among $CD4^{pos}$ $FoxP3^{pos}$ Tregs has been associated with their suppressive function by inducing adenosine, which is an immunoinhibitory molecule (Borsellino et al., 2007; Deaglio et al., 2007; Fletcher et al., 2009). Most importantly it has been postulated that Tregs that up-regulate CD39 may represent the memory subset required for long-term immunoregulation (Zhou *et al.*, 2009b). Likewise, effector T cells highly up-regulated CD39 expression and barely any $CD39^{neg}$ population was detected upon co-cultures with pCB Tregs. Thus, in agreement with other studies, CD39 expression is not exclusive of Tregs, however, its expression on Tregs is associated with their suppressive functionality as well as with their differentiation to a memory Treg subset (Zhou *et al.*, 2009b). In contrast, CD39 expression on effector T cells only represents the latter. More details regarding CTLA-4 and LAP expression on Tregs will be described in details in Chapter 6.

A)



B)

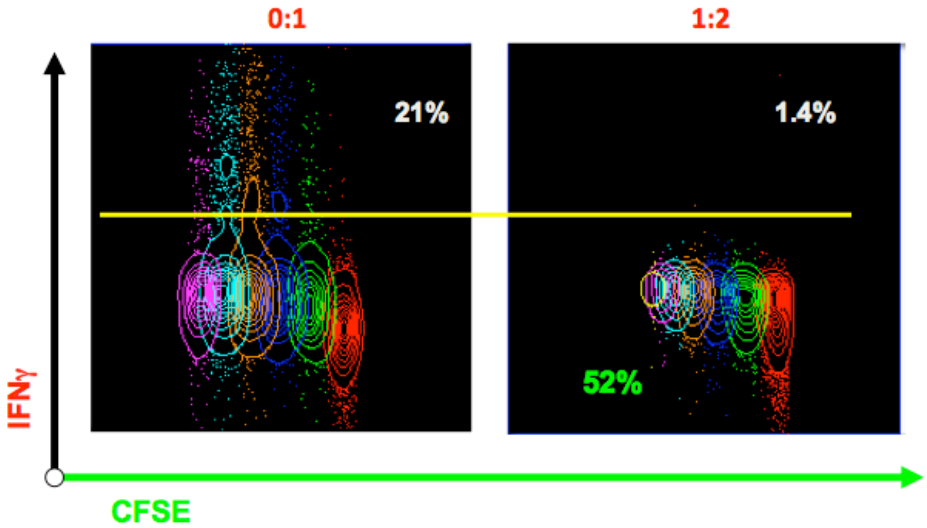


Figure 5.7 Representative suppression assay using CFSE staining (n=4). A) Histograms gated from CFSE+ population. Upper panel shows expression of activation markers (HLA-DR, CD69 and CD25), lower panel shows markers related to Treg function (CTLA-4, LAG-3 and LAP). Red color histogram depicts 0:1 and **Gray** depicts 1:2 Tregs: effector cells ratio. **B)** Overlay counter plot graph showing cell divisions in different colors (gated from CFSE+ population). Left panel shows 1:0 and right panel 1:2 Tregs: effector cells ratio. Percentage shown in white represents the level of IFN-γ secretion. Percentage of **suppression** is shown in **green.**

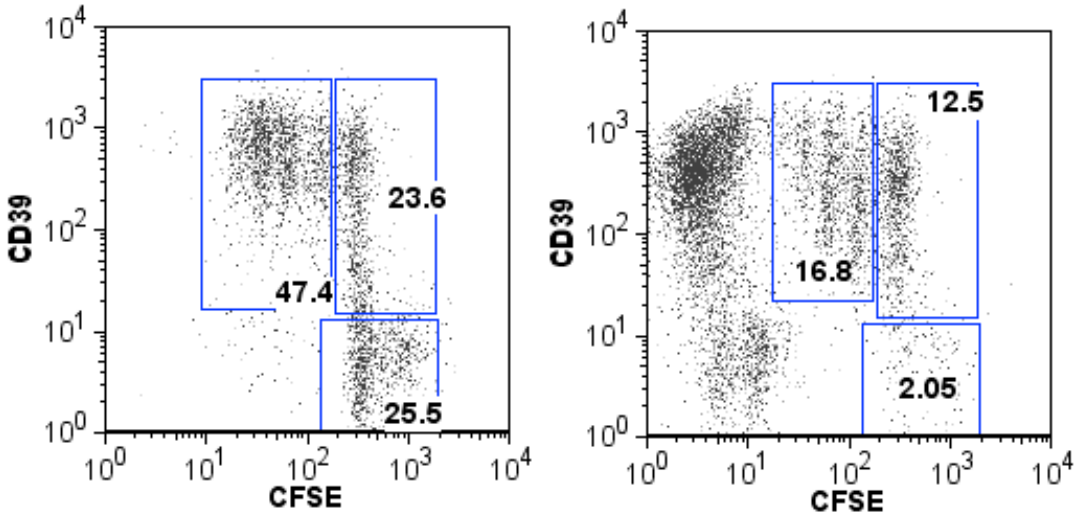


Figure 5.8 Representative suppression assay measuring CD39 expression (n=3). Dot plot graph showing CFSE dilution compared to CD39 expression. Left panel shows 0:1 and right panel 1:2 Tregs: effector cells ratio. Gates with percentages illustrates CD39 expression on non-proliferating and proliferating effector T cells.

5.2.5 pCB Tregs suppression is cell-contact independent.

To further test the capacity of pCB Tregs suppression and to elucidate if they require cell contact to regulate, MLCs (n=6) were done in 24 and 96 transwell plates. pCB Tregs were added either in the upper or lower chamber in a 1:1 Tregs:effector cell ratio. pCB Tregs showed a potent suppression in a cell contact independent manner (Figure 5.9). Moreover, pCB Tregs did not require TCR engagement from the stimulator cells (irradiated PBMCs) to be suppressive. The same level of suppression was observed when pCB Tregs were co-cultured or not with irradiated PBMCs in the upper chamber. However, it is a common consensus that Tregs require TCR engagement for them to be suppressive (Thornton *et al.*, 2000). More recently, it has been shown in a mice model using transgenic TCR Tregs that suppression can be achieved without Tregs TCR engagement (Szymczak-Workman et al., 2009). In addition, it has

also been shown that T cells can interact with other T cells, also called “T-T interactions” (Helft *et al.*, 2008; LaSalle *et al.*, 1992; Lee *et al.*, 2009; Sidhu *et al.*, 1992; Tsang *et al.*, 2003). In accordance, and due to the general consensus that nTregs are considered to be an already pre-activated population as they exit the thymus, that, in addition to their expression of MHC-II and the specific fact that allorecognition is induced by pooling a variety of mismatch fully capable $CD4^{pos}$ T cells with a wide TCR repertoire, would confer the settings and the capacity for TCR interaction between each other.

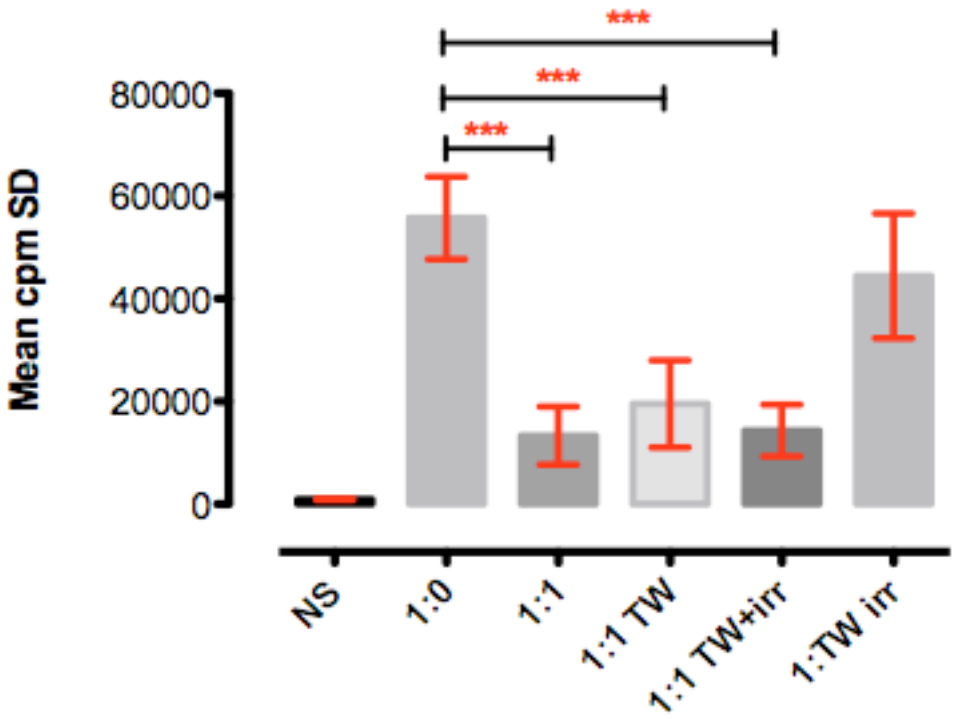


Figure 5.9 Representative Trans-well suppression assay (n=6). Normal MLCs were done in triplicates with the following settings**: 1:0=** Normal MLC in the lower chamber, **1:1=** Tregs:effector cells ratio co-cultured in the lower chamber, **1:1 TW=** irradiated PBMC and responders in the lower chamber and pCB Tregs in the upper chamber ending in 1:1 Tregs:effector cells ratio, **1:1 TW+irr=** the same as the previous with the addition of irradiated PBMC in the upper chamber with the pCB Tregs and **1:TW irr=** normal MLC in the lower chamber and irradiated PBMCs in the upper chamber. Results are shown as Mean cpm ± SD. A One-way ANOVA with Tukey’s post-test was done for statistical analysis (*** ***p*=<0001**).

5.2.6 pCB Tregs can be used as an adjuvant to host Tregs.

Current data regarding Treg immunotherapy for allo-transplantation suggest that specificity as well as cell numbers are important elements for effective suppression in vivo (Hoffmann et al., 2006a; Riley et al., 2009; Sagoo et al., 2008). In agreement to this, I tested the feasibility of using host Tregs in addition with pCB Tregs, hereafter mentioned as host/CB pool. MLCs were carried out as described previously and Tregs were added at different Tregs:effector cell ratios. Host/CB pool was added only at a final 1:2 Treg:effector cells ratio. Within the host/CB pool, only 20% or 10% were host Tregs, with the rest being pCB Tregs. Interestingly, host/CB pool showed the same level of suppression compared to using only host Tregs. This effect was conserved even with the lowest proportion of host Tregs (10% host Tregs + 90% pCB Tregs) (Figure 5.10). As it has been shown, third party adult Tregs showed lower level of suppression compared to host Tregs (p=0.001). However, pCB Treg showed potent suppression with overall no statistical significance compared to host Tregs. As described on Chapter 3, CB Tregs are mainly naïve (~89%, $CD45RA^{pos}$) and most of them express CD31 (~80%), which is a marker correlated with recent thymic emigrants with a wide TCR repertoire (Junge et al., 2007). Conversely, adult Tregs are mainly central memory ($CD45RA^{neg}$, $CD62L^{pos}$), with <15% being $CD45RA^{pos}$ $CD31^{pos}$. These phenotypic disparities among adult and CB Tregs could explain the different level of suppression achieved when used as “third party” in allo-responses.

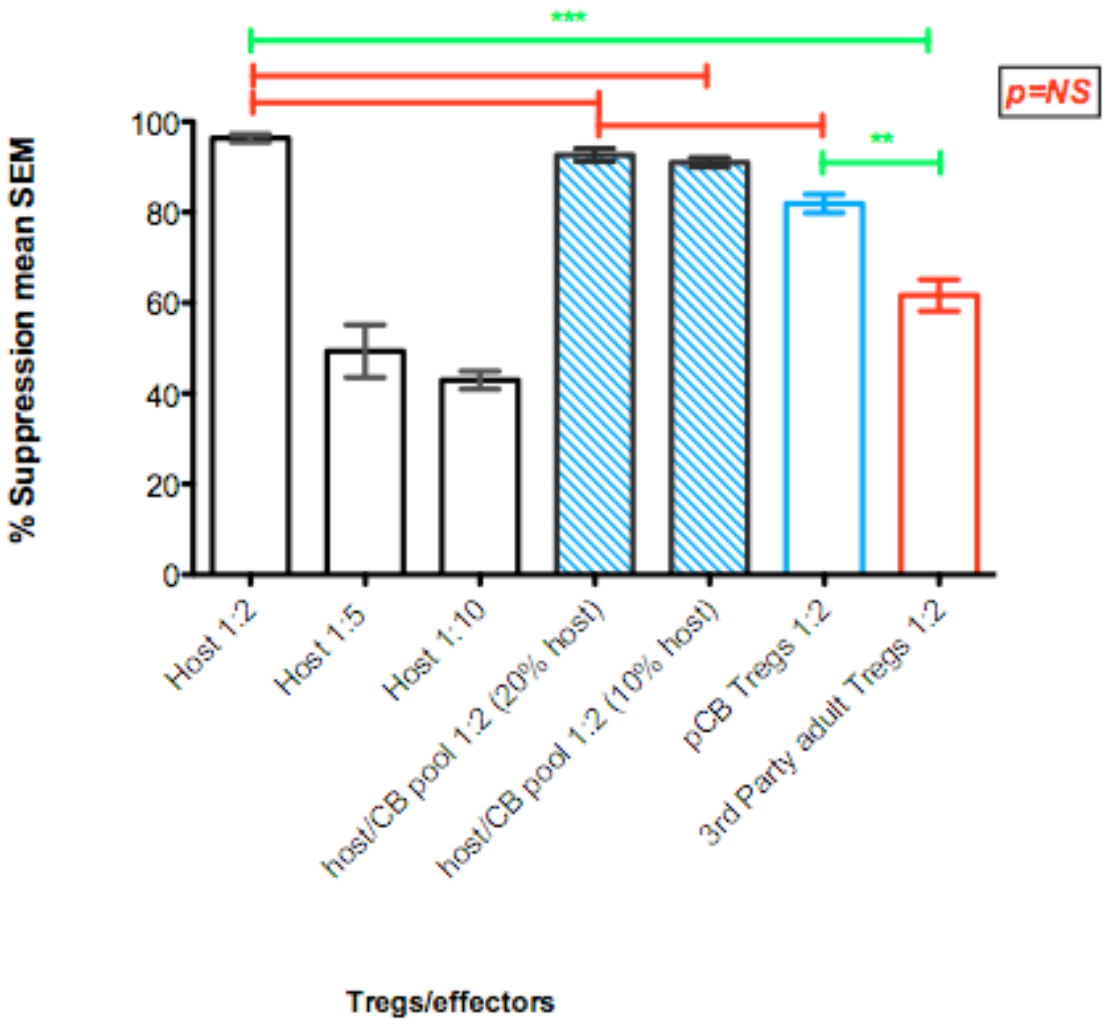


Figure 5.10 Summary of suppression assays using pCB Tregs + host Tregs (n=3). Results are shown as mean % of suppression ± SEM. Host Tregs were added at 1:2, 1:5 and 1:10 Tregs: effector cell ratios. Host/CB pool (pCB Tregs +host Tregs) was added at 1:2 Tregs:effector cells ratio with 20% and 10% being host Tregs, respectively. pCB Tregs and adult third party Tregs were added at 1:2 Tregs:effector cells ratio. **Green** lines, shows statistical differences (*******p*=0.001*****p*=<0.0001**) and red lines no statistical difference (**NS**). A One-way ANOVA with Tukey's post-test was done for statistical analysis.

5.2.7 pCB Tregs immunogenicity

One of the main concerns regarding the use of third party cellular components in allo-transplantation may be increasing alloreactivity that may end up with a non-beneficial effect to the host or to the graft. To address this issue, pCB Tregs were irradiated as described in section 2.5.1 either freshly isolated or after 48hrs of activation with soluble CD3/CD28 Ab. The latter was done to elicit higher expression of MHC II from pCB Tregs. Cultures were done in triplicates and follow up as a standard MLC. As shown on Figure 5.11, irradiated pCB Tregs did not elicit proliferation of $CD4^{pos}$ CD25neg T cells. The same results were shown when used activated pCB Tregs.

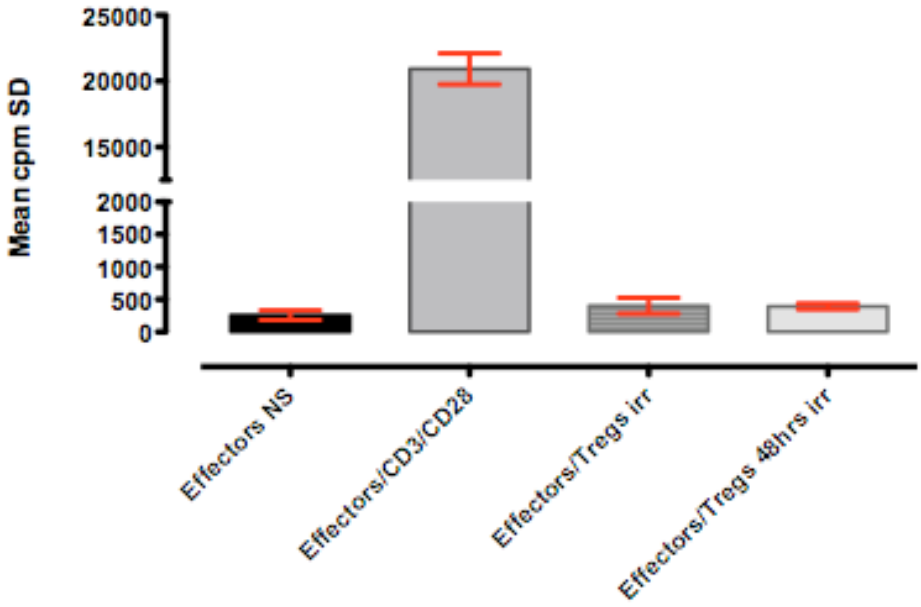


Figure 5.11 pCB Tregs immunogenicity assay (n=2). Results are shown as mean cpm ±SD. $CD4^{pos}$ $CD25^{neg}$ T cells (effectors) stimulated either with soluble anti-CD3/CD28 Ab or irradiated $1x10^5$ pCB Tregs (freshly isolated or 48hrs post activation).

5.3 Discussion

It has been widely shown that host or donor Tregs, are the most potent suppressors for allo-responses (Hoffmann *et al.*, 2006c; Sagoo *et al.*, 2008). On my understanding, the third party approach has not been fully explored. Few studies have actually used third party Tregs to suppress alloresponses and the vast majority have tested either donor or host Tregs expanded against third party antigens. Perhaps the caveats of some of these experiments could be due to the fact of using adult Tregs that in contrast to the murine models are mainly memory Tregs that have been induced within a heterologous immune system. In addition, expanding these memory Tregs towards alloantigens would imply a second selection process, which will inherently narrow the TCR repertoire of the ending Treg pool, hence lesser chances of suppressing alloresponses. This could explain the low suppressive potency of third party expanded Tregs shown in these reports and can also mislead the actual approach of using Tregs from a third party. In contrast, the only studies to my knowledge, using Tregs from a third party that have shown potent suppression of alloresponses, comes from a murine model study (Steiner et al., 2006) and others using human CB Tregs (Godfrey et al., 2005; Hippen et al., 2008). In accordance, herein is shown that CB Tregs are optimal for a third party approach. In addition, compelling data demonstrates that $CD45RA^{pos}$ Tregs constitute the most homogenous and stable population among the overall Treg pool (Hoffmann et al., 2006b; Miyara et al., 2009). However, only a small proportion of adult Tregs are $CD45RA^{pos}$ (~20%). In contrast, CB Tregs are mainly $CD45RA^{pos}$ (~89%) and co-express CD31 which is a marker correlated with recent thymic emmigrants which also maintain a wide TCR repertoire (Kohler *et al.*, 2008). As it was shown, CB Tregs

constitutively suppressed direct allo-recognition. Although a noticeable better suppression was detected using host Tregs, there was no statistical difference detected when compared to overall CB Tregs suppression. In contrast, a lesser level of suppression was seen when adult Tregs were used as third party.

pCB Tregs showed in the same manner, constitutively potent suppression ability in MLC. Most importantly, pooling did not affect their suppressive capacity. Moreover, in 40% of the cases a better suppression was seen with pCB Tregs compared to individual CB Tregs suggesting a "synergy effect". Noteworthy, no HLA matches (HLA-A,B,C and DR) were detected between the CB units pooled, responders and stimulators used in the MLCs. These results suggest that pooling could increase the repertoire for allo-recognition, and thus, induced better suppression.

Lower levels of suppression were detected in a non-APC system. Effector T cells were able to up-regulate activation markers when co-cultured with pCB Tregs. However, pCB Tregs potently inhibited the differentiation of $CD4^{pos}$ $CD25^{neg}$ cells into cytokine secreting Th-1 cells, as they potently blocked IFN-γ secretion. Interestingly, a higher percentage of effector cells became LAP^{pos} when co-cultured with pCB Tregs. It has been shown that iTregs can be developed from activated $CD4^{pos}$ $CD25^{neg}$ T cells in the presence of TGF-β and upon activation they constitutively up-regulate LAP expression (Chen et al., 2003).

Furthermore, pCB Tregs highly up-regulated CD39, which has been shown to work in tandem with CD73 for their suppressive function (Borsellino et al., 2007;

Fletcher et al., 2009). In addition, since CD39 expression also allows Tregs to be resistant to apoptosis, it has been postulated that CD39 expression on Tregs, is of great importance for the development of a memory pool to achieve long-term immunoregulation (Zhou *et al.*, 2009b).

It has been widely believed that Tregs requires TCR engagement for proper functionality (Thornton *et al.*, 2004b). pCB Tregs did not require cell contact with the stimulator cells in order to be suppressive. It has recently been shown suppression without the requirement of prior TCR engagement using transgenic TCR Tregs (Szymczak-Workman et al., 2009). Moreover, it has also been shown that T cells can get TCR engagement between T cells, called "T-T interactions" (Helft *et al.*, 2008; LaSalle *et al.*, 1992; Lee *et al.*, 2009; Sidhu *et al.*, 1992; Tsang *et al.*, 2003). Since, CB Tregs constitutively express MHC II and conserve a naïve wide TCR repertoire, and the specific fact that allorecognition is induced by pooling, it is more likely that T-T interactions may allow CB Tregs suppression without the traditional engagement within a T cell and an APC. Interestingly, this finding suggests that the process of *pooling* can elicit a higher Treg pool with already TCR engagement. This could be of great importance for their suppressive functionality once infused *in vivo*. Moreover, the majority of the experiments *in vitro* have shown that Tregs requires cell-contact to be suppressive (Shevach, 2009). Interestingly, pCB Tregs showed suppression in a cell contact independent manner. It has been described that Tregs can mediate suppression through the secretion of inhibitory cytokines, mainly TGF-β, IL-10 and recently IL-35 (Asseman et al., 1999; Nakamura et al., 2004; Vignali et al., 2008).

From an *in vivo* model experiment, using cord blood polyclonal expanded Tregs, higher ratios (4:1 to 6:1 Tregs:effectors) are required to effectively suppress allo-responses (Hippen et al., 2008). However, a lower purity of Tregs was used in this experiment, therefore, it is hard to elucidate if that higher dose is really required. Nonetheless, further experiments are still warranted to identify the optimal time for the adoptive transfer of Tregs, since this is an important factor that might influence greatly the cell dose required. Likewise the necessity of multiple infusions is also another option that has not been elucidated. pCB Tregs showed to be a practical approach that could achieve optimal cell numbers with GMP standards using a single step isolation method when requested. They constitutively suppress allo-responses *in vitro*. Furthermore, pCB Tregs showed potent suppression ability when used as adjuvant to host Tregs suppression.

It has been proven in preclinical models that donor polyclonal expanded Tregs are efficient to prevent GvHD in HSCT (Edinger et al., 2003). In contrast, it has been shown that Tregs with direct-alloantigen and indirect-alloantigen specificity are required for long-term graft survival in solid organ transplantation settings (Joffre et al., 2008; Tsang et al., 2008). However, the time period required in order to achieve optimal cell numbers with the specificity required in both clinical settings may not be feasible in time constrain scenarios. In addition, long-term (>2 weeks) Tregs expansion protocols have shown down-regulation of FoxP3 expression and conversion/outgrowth to Th-1, Th-2 or Th-17 effector T cells (Hoffmann et al., 2009; Putnam et al., 2009). Therefore, pCB Tregs can be used

in addition to host Tregs expanded for shorter time periods in order to achieve optimal cell numbers for immunotherapy. This approach can address specificity and optimal cell numbers in a practical and safe fashion, and even help to save some additional host or donor Tregs, that might be required for multiple infusions.

Irradiated pCB Tregs did not elicit any proliferation from effector T cells, further experiments using *in vivo* models are warranted to fully corroborate these results.

CHAPTER 6

pCB TREGS ACTIVATION PATTERN AND REGULATORY PHENOTYPE STABILITY

6.1 Introduction

Evolving studies have correlated a wide repertoire of markers with Tregs differentiation, activation and their functional properties (Brusko *et al.*, 2008b). These results have provided valuable data in order to elucidate a *bona fide* Treg for their proper translation into clinical settings.

The majority of nTregs in the peripheral blood constitutively express high levels of the IL-2α receptor (CD25) (Baecher-Allan et al., 2001). It has also been described that the IL-2 second signal after TCR engagement, is of importance for Tregs differentiation in the thymus (Fontenot *et al.*, 2005a). Furthermore, activated STAT5, downstream of IL-2 receptor signalling, regulates FoxP3 transcription (Burchill et al., 2007; Wuest et al., 2008). Therefore, IL-2 is paramount for Tregs differentiation and phenotype stability. In addition, CTLA-4 is constitutively expressed on nTregs (Baecher-Allan et al., 2001; Miyara et al., 2009; Read et al., 2000), and it has been suggested to be a core mechanism through which nTregs harness APC function (Friedline et al., 2009; Shevach, 2009). nTregs also express the glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR). Signalling through this receptor abrogates nTregs suppressive capacity (McHugh *et al.*, 2002; Nocentini *et al.*, 2005; Shimizu *et al.*, 2002). Moreover, the expression of the lymphocyte activation

gene-3 (LAG-3) on Tregs, which is a homologue of CD4, modulates APCs function by interacting with their MHC II molecules (Liang et al., 2008). TGF-β plays an important role within the repertoire of Tregs cytokine-mediated suppression mechanisms (Huber *et al.*, 2006; Nakamura *et al.*, 2004; Nakamura *et al.*, 2001). Usually TGF-β is detected in the inactive form associated with latency-associated peptide (LAP) and is constitutively expressed on activated Tregs with potent suppressive function (Nakamura et al., 2004; Tran et al., 2009a; Tran et al., 2009b). It has also been described that the expression of different homing receptors like CD62L and CCR7 on Tregs, are predominant for their ability to migrate to different areas of importance within the body in order to suppress (Taylor et al., 2004; Zhang et al., 2009). In accordance, CXCR3 signalling favours Tregs recruitment and interactions with T effector cells within Th-1 cell-mediated inflammation sites (Muller et al., 2007).

Lastly, the awareness of possible Tregs plasticity (or outgrowth of the contaminating effector T cells) towards unwanted effector T cells (Th-1, Th-2 and Th-17) is a main concern for the proper translation of Treg immunotherapy into clinical settings (Afzali et al., 2010; Hoffmann et al., 2009; Putnam et al., 2009; Riley et al., 2009). Therefore, in addition to the functional assays that measure suppressive potency, different tests are warranted to confirm the regulatory phenotype stability of the cells intended to infuse.

6.2 Results

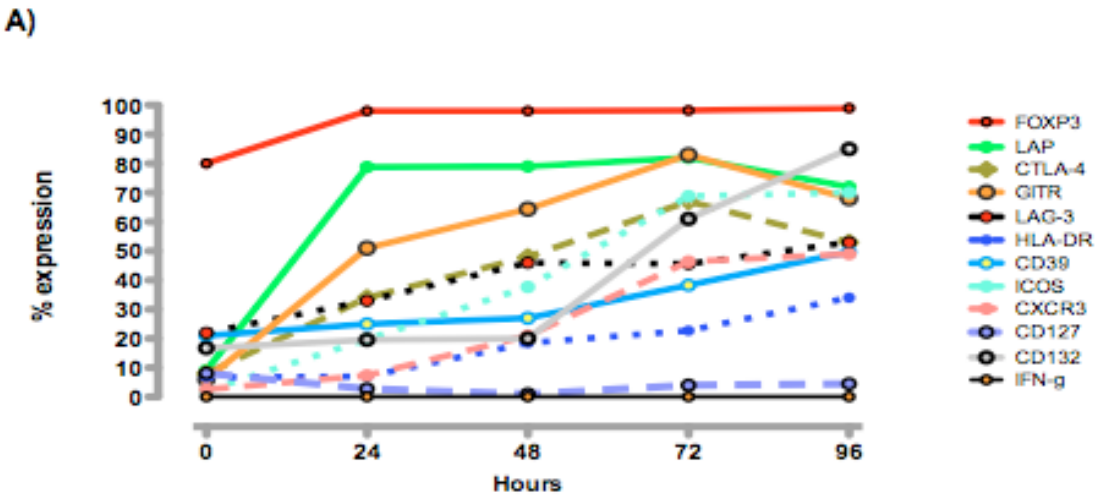
In accordance to the aforementioned, the objective of this chapter is to measure the expression of different markers upon Tregs activation. This will allow us to clarify the changes in pCB Tregs following activation. pCB Tregs will be activated using polyclonal stimulation, this with the only intention to avoid the variability that will be inherent otherwise an allo-stimuli is used. In addition, pCB Tregs will be tested upon different conditions to ascertain their regulatory phenotype stability.

6.2.1 pCB Tregs activation pattern is consistent with Treg phenotype and functionality.

pCB Tregs were cultured with polyclonal stimuli for 96hrs in order to observe the initial activation process of pCB Tregs and whether they were able to convert or restrict the outgrowth of the initial contaminating effector T cells (~9%). In accordance, pCB Tregs up-regulated the wide repertoire of markers correlated with Treg phenotype and functionality (Figure 6.1 **A,B**). FoxP3 expression was upregulated within the first 24hrs (from ~80% to ≥90% $CD4^{pos}$ $FoxP3^{pos}$) of culture and hereafter, highly maintained throughout the culture (>90% $CD4^{pos}$ $FoxP3^{pos}$). LAP expression was up regulated within the first 24 hrs and maintained the level of expression (~80% $CD4^{pos}$ LAP^{pos}), with an apparent downslope after 72hrs of culture (72% $CD4^{pos}$ LAP^{pos}). The expression of CTLA-4 and GITR showed a slightly slower pace of up-regulation, with the highest expression achieved (67% $CD4^{pos}$ CTLA-4^{pos} and 83% $CD4^{pos}$ $GITR^{pos}$) at 72hrs of culture. The same activation pattern was seen for the expression of LAG-3,

HLA-DR, CXCR3, CD39 and ICOS. Lastly, pCB Tregs highly upregulated the expression of the γ_c chain receptor CD132 (>80%). It has been shown that Tregs requires the three components of the IL-2 receptor, which are CD25 (IL-2Rα), CD122 (IL-2Rβ) and CD133 (IL-2Rγ_c), for their functionality and homeostasis in vivo (Fontenot *et al.*, 2005a; Thornton *et al.*, 2004a; Yu *et al.*, 2009a).

In summary, pCB Tregs upregulated all the characteristic markers consistent with a regulatory phenotype that are important for their suppressive functionality. In accordance lower levels of CD127 (<4% $CD127^{pos}$) were maintained throughout the culture and no IFN-γ secretion was detected. Therefore, under this conditions pCB Tregs conserved a stable regulatory phenotype with an apparent control over the initial effector T cell population.



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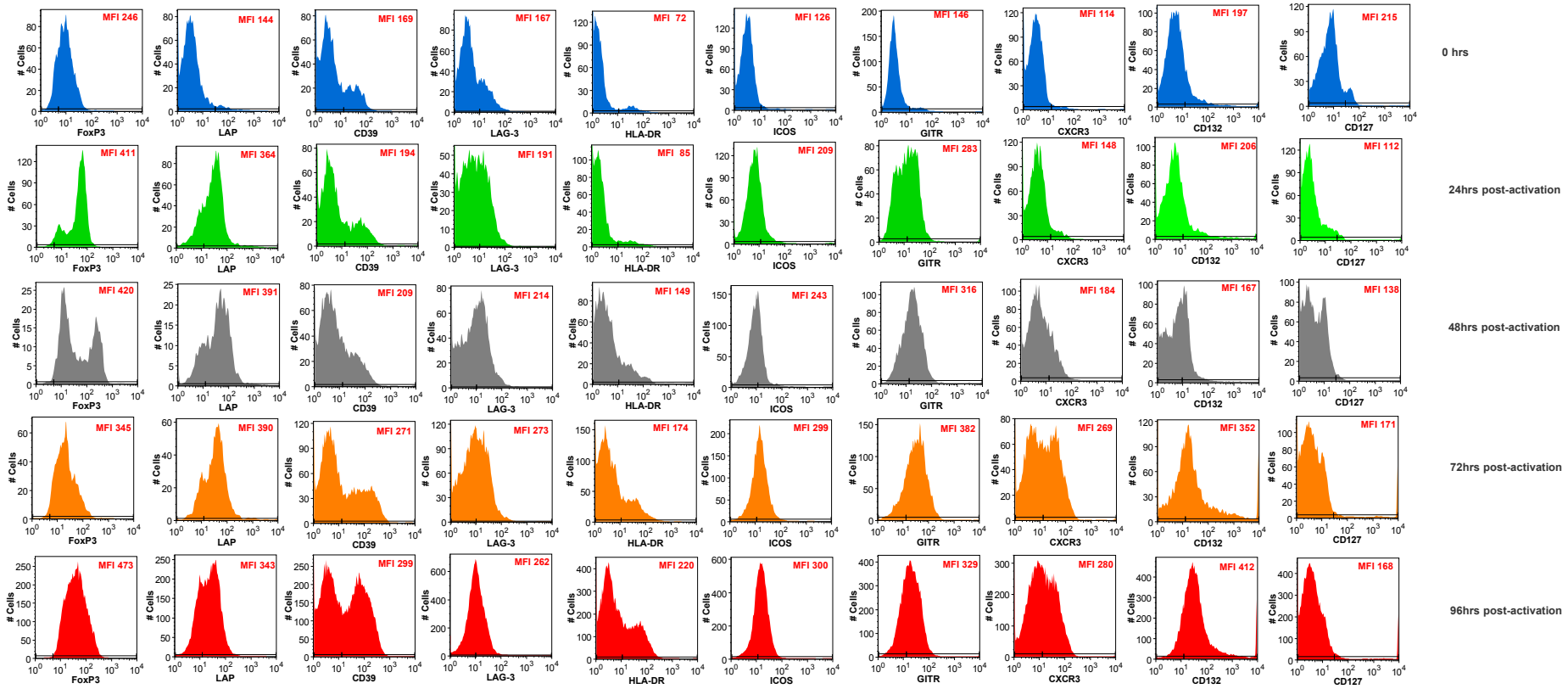


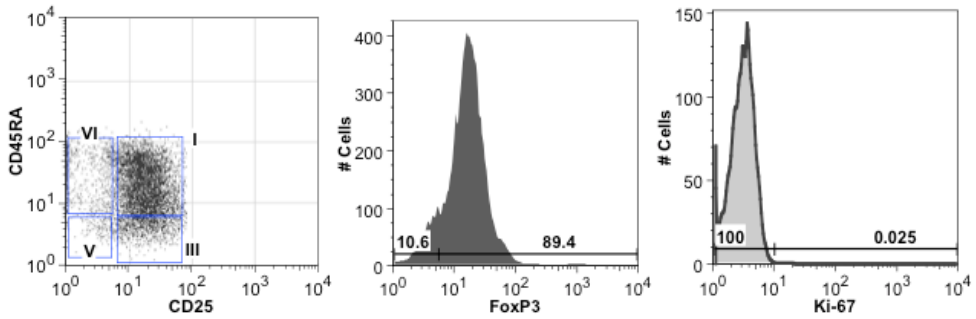
Figure 6.1 pCB Tregs activation pattern (n=2). A) pCB Tregs were activated with soluble CD3/CD28 antibodies. The expression of different markers, were measured at the estimated time points. Results are shown as percentage of expression gated from $CD4^{pos}$ cells. Positive fraction of each marker was depicted using the specific isotype control as instructed in Material and Methods Chapter.
B) Histograms of the different markers at the designated time points are shown. Results are shown as mean intense fluorescence intensity (MFI).

6.2.2 pCB Tregs differentiate upon activation from rTregs to aTregs with no sign of conversion/outgrowth to Th1, Th2 and Th-17.

In order to test in a longer time frame the differentiation and stability of pCB Tregs, the cells were placed in 7 days culture with polyclonal activation using CD3/CD28 beads (1:2 bead:cell ratio) in addition to 400U/ml of IL-2, then rested for 24hrs in culture media for a posterior activation with PMA/Ionomycin. In agreement with a previous study (Miyara et al., 2009), in which rTregs were characterized as $CD45RA^{pos}$ $FoxP3^{low}$ (prior culture ~80% vs post activation <20%) became in their vast majority aTregs $CD45RA^{neg}$ $FoxP3^{high}$ (prior culture ~12% vs post activation >65%, Figure 6.2 **A**,**B**). The expression of Ki-67, which is a nuclear marker used for proliferation (Scholzen *et al.*, 2000), was not depicted from overall $CD4^{pos}$ T cells from fresh CB (Figure 6.2 **A**). After activation of pCB Treg, Ki-67 expression was mostly up-regulated in the $FoxP3^{pos}$ population compared to the $FoxP3^{neg}$ (>30% vs <2% $Ki67^{pos}$, respectively). In addition Ki-67 expression, among the $FoxP3^{pos}$ cells, was nonexclusive of their CD45RA expression. Ki-67 expression was expressed in the same proportion on the $CD45RA^{pos}$ and $CD45RA^{neg}$ $FoxP3^{pos}$ T cells, suggesting a homeostatic balance between the overall Treg pool.

Lastly, after a week of strong activation, pCB Tregs did not show any production of IFN-γ (Th-1), IL-4 (Th-2) or IL-17a (Th-17) which suggests a lack of conversion or outgrowth towards unwanted effector T cells (Figure 6.2 **C**). Additionally, no IL-10 production was observed.

A)



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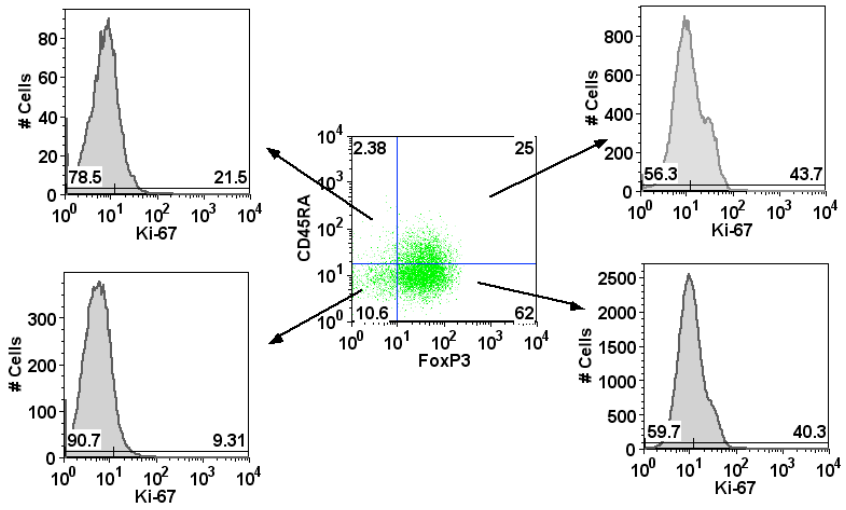

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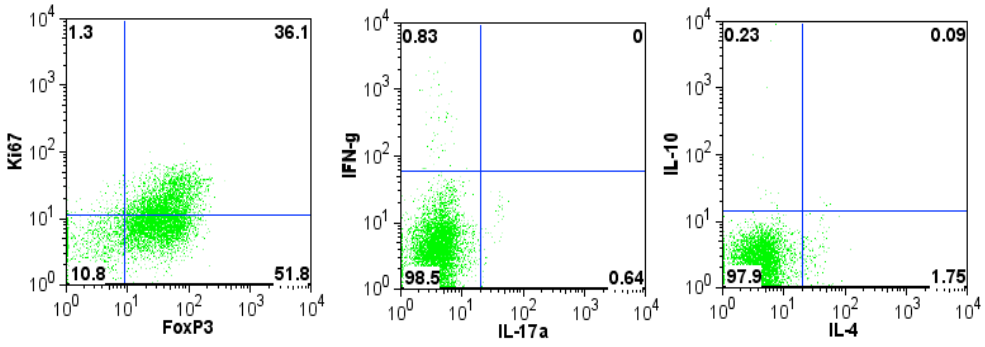


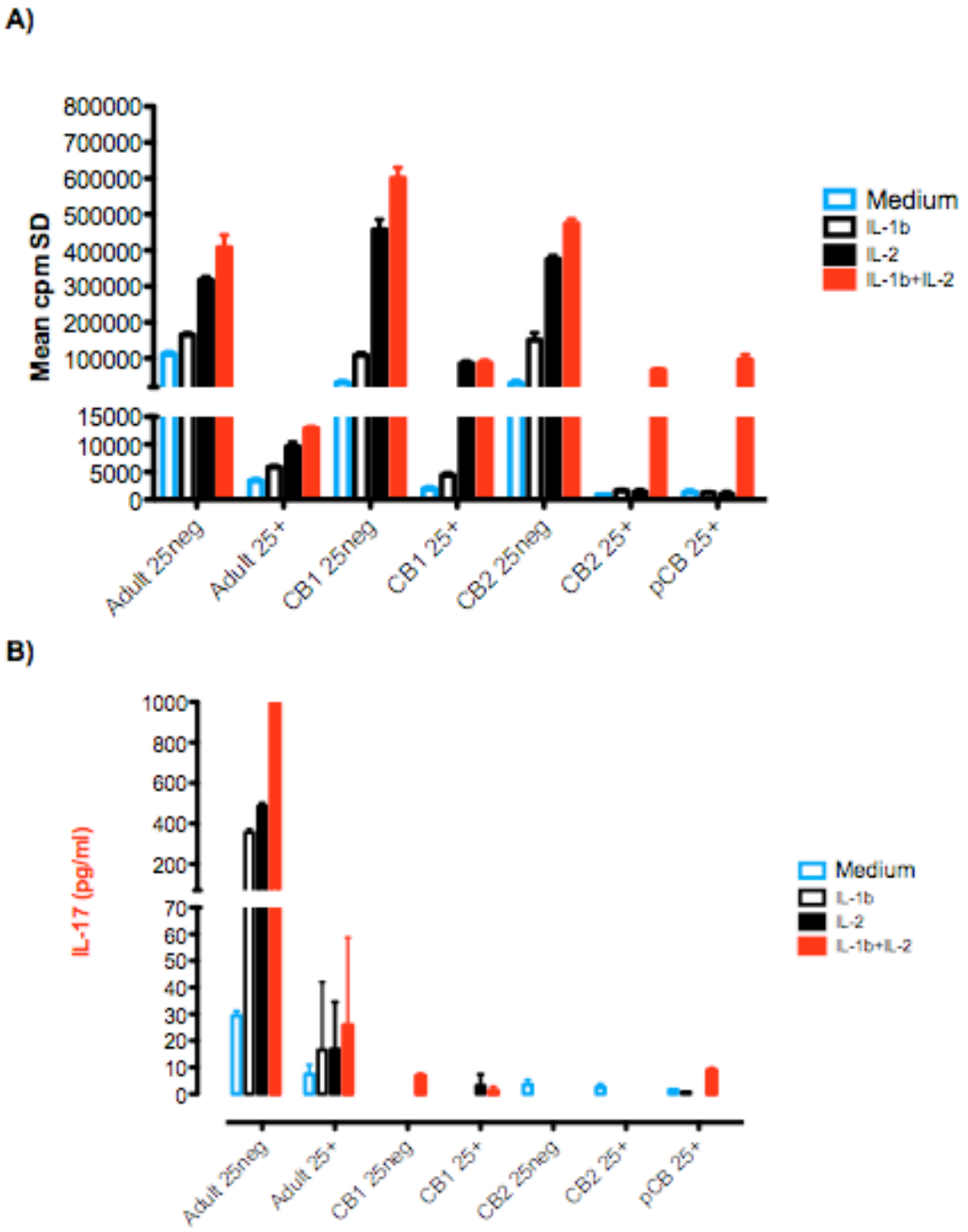
Figure 6.2 Representative activation culture using CD3/CD28beads and PMA/Io of pCB Tregs (n=3). Results are shown as percentage of expression gated from $CD4^{pos}$ population. A) Dot plots and histograms shows the initial purity and characterization of pCB Tregs using FoxP3, CD25, CD45RA and Ki-67. Gate I depicts rTregs and gate III shows aTregs (gates V and VI determine effector cells). B) Dot plots and histograms of pCB Tregs after activation showing percentage of expression of FoxP3, CD45RA and Ki-67. C) Dot plots of pCB Tregs after activation showing percentage of Ki-67 and FoxP3 and intracellular cytokine staining for IFN-γ, IL-4, IL-10 and IL-17a.

6.2.3 Overall CB $CD4^{pos}$ T cells are reluctant to Th-17 differentiation under the influence of IL-1b and IL-2.

It has been shown that IL-1b and IL-2 can elicit Treg conversion towards Th-17 cells (Beriou et al., 2009; Deknuydt et al., 2009). Therefore, to test this, adult $CD4^{pos}$ $CD25^{pos}$, $CD4^{pos}$ $CD25^{neg}$ T cells, CB $CD4^{pos}$ $CD25^{pos}$, CB $CD4^{pos}$ $CD25^{neg}$ and pCB Tregs were challenged by polyclonal activation using CD3/CD28 beads (1:2, bead:cell ratio) in the addition of IL-1β and/or IL-2 (10ng/ml and 10U/ml final concentration, respectively) for 5 days. Proliferation was measured using thymidine incorporation, and the levels of IL-17, were measured from the supernatant (using ELISA, described in section 2.7).

As expected, $CD4^{pos}$ $CD25^{neg}$ subsets from adult and CB showed higher proliferation at the end of culture compared to their counterpart $CD25^{pos}$ population. The addition of IL-1b and IL-2 into culture elicited higher proliferation rate compared to their individual effect upon each cell subtype from both sources (adult and CB, Figure 6.3 A). This finding also validates the viability of each cell subset.

Overall, IL-17 was mostly detected from adult $CD4^{pos}$ cells compared to $CD4^{pos}$ from CB. Either IL-1b or IL-2 generated the secretion of IL-17 from both $CD25^{neg}$ and $CD25^{pos}$ $CD4^{pos}$ T cells in adult. The mixture of IL-1b and IL-2 within the same culture showed the best milieu for IL-17 secretion from both cell subtypes in adult. Conversely, overall CB $CD4^{pos}$ T cells showed barely any levels of IL-17 hence no difference could be detected between individual CB $CD4^{pos}$ cell subsets or pCB Tregs (Figure 6.3 B).



6.3 Representative activation of CB and adult CD4^{pos} cell subsets with IL-1b or/+ IL-2 (n=2) CD25^{pos} and negative fractions from CD4^{pos} cells of adult and CB were activated with CD3/CD28 beads (1:2 bead:cell ratio) with the addition of the described cytokine/s. **A)** Graph showing proliferation of each cell subtype with the described cytokines in culture. Results are shown as mean cpm±SD. **B)** Graph showing the level of IL-17 captured from the supernatant from each cohort as described. Results are shown as mean pg/ml ±SD.

6.2.4 Paucity of Th-17 conversion/outgrowth from expanded pCB Tregs, whilst maintaining their suppressive capacity.

As mentioned before, Tregs expansion protocols have shown some important levels of conversion or outgrowth towards Th-1 or Th-17 effectors (Beriou et al., 2009; Putnam et al., 2009; Riley et al., 2009). Herein, I showed that after long-term culture (21 days), which will infer 3 steps of stimulation with CD3/CD28 beads, pCB Tregs were able to expand (45 to 60 folds, Figure 6-4 **A**). However, around 10% of the $FoxP3^{pos}$ population showed IL-17a production with no level of IFN-γ or IL-10 depicted (Figure 6-4 **B**). Noteworthy, the initial purity was ~90% for $CD4^{pos}$ $FoxP3^{pos}$, therefore, it is more likely that the 10% of $CD4^{pos}$ contaminating effector cells may be responsible for the level of IL-17a depicted. However, strict elucidation between conversion or outgrowth of Th-17 effector cells can not be assessed from this experiment. Nonetheless, expanded pCB Tregs maintain their suppressive capacity (Figure 6.4 **C**).

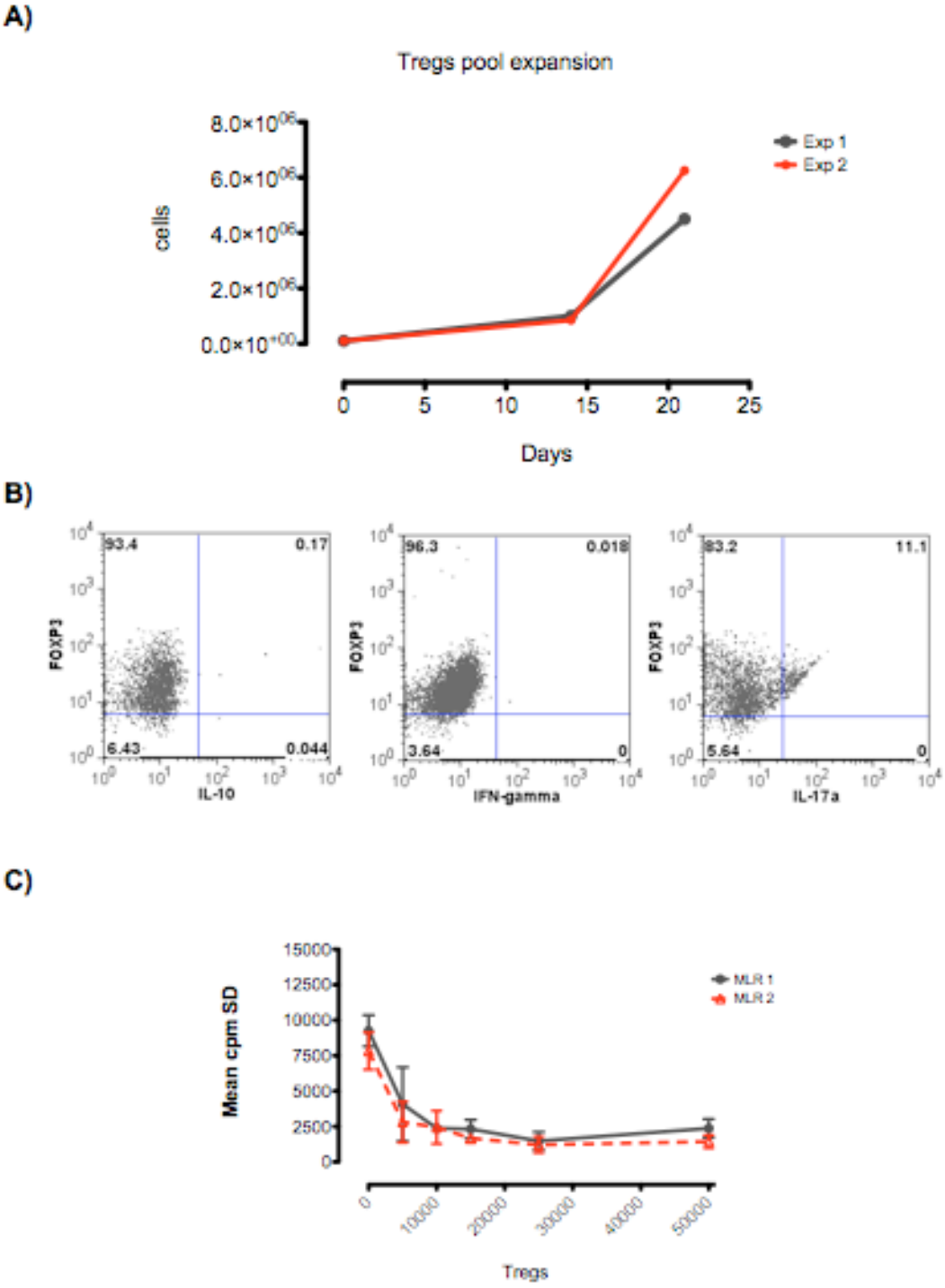


Figure 6.4 pCB Tregs expansion (n=2) A) Expansion of (1x10^5 cells) pCB Tregs with CD3/CD28 beads (1:2, bead:cell ratio) with 400U/ml of IL-2. Results are shown as total number of cells. **B)** Characterization of pCB Tregs at 21 days of culture. Results are shown as percentage of expression gated from CD4^{pos} cells. **C)** Suppression assay (MLC) using the expanded pCB Tregs at different cell numbers as described. Results are shown as mean cpm ±SD.

6.3 Discussion

Compelling data has shown that CD45RA^{pos} FoxP3^{pos} Tregs also called rTregs, represent the most homogenous and stable population of the overall Treg pool (Hoffmann *et al.*, 2006b; Miyara *et al.*, 2009). In agreement, pCB Tregs, which are in there vast majority rTregs (>80%), behave as such upon activation. They showed a homeostatic proliferation balance upon activation, ending up with a reduced rTregs Ki-67^{pos} pool and an increased population of aTregs. However, in contrast to Miyara's study, the expression of Ki-67 on overall CB Tregs is only depicted once being activated, suggesting that overall naïve CD4 T cells from CB are in a clearly quiescent state in comparison to their similar population from adult PBMCs. Additionally, pCB Tregs upon polyclonal stimuli, constitutively up-regulate and maintain their FoxP3, and GITR expression without any production of IFN-γ or up-regulation of CD127 that would imply any effector T cell differentiation or outgrowth. Moreover pCB Tregs highly upregulate LAP expression. A similar pattern of LAP expression has been reported from sorted adult CD4^{pos} CD25^{high} T cells (Tran et al., 2009a). In this study they showed that an additional isolation of LAP^{pos} cells specifically depicts activated CD4^{pos} FoxP3^{pos} T cells with potent suppressive capacity. In addition, pCB Tregs up-regulate CTLA-4, which has been shown to be important inhibiting the inflammatory cascade through cell contact with CD80/86 expressed on APCs (Onishi et al., 2008). CTLA-4 signal can activate indoleamine-2,3-dioxygenase (IDO), which generates the immunosuppressive mediator kynurenin (Grohmann et al., 2002). Moreover, signalling through CTLA-4 promotes the nuclear localization of Foxo transcription factors, which hampers the expression of genes encoding IL-6 and the phosphorylation of the tumor necrosis factor

(Dejean et al., 2009). Therefore, CTLA-4 expression by Tregs is of great importance for their function *in vivo*. pCB Tregs also express LAG-3, which has been shown that $CD4^{pos}$ T cells transduced with LAG-3, confers them regulatory ability (Huang et al., 2004). Moreover, dendritic cell maturation and activation is hampered by the engagement between their MHC II molecules and LAG-3 from Tregs (Liang et al., 2008).

As previously mentioned (Chapter 3), another important characteristic of Tregs is their capacity to migrate to sites of relevance of the immune response in hand, specifically the target tissue and the secondary lymph nodes. Accordingly, pCB Tregs constitutively express CD62L and CCR7, which are important for their ability to migrate to secondary lymph nodes, wherein they can inhibit APCs maturation and activation (Taylor et al., 2004; Zhang et al., 2009). Moreover, it has been shown that Tregs that upregulate the transcription factor T-bet and subsequently the expression of CXCR3, specifically have the capacity to migrate and suppress at sites of Th-1 cell-mediated inflammation (Koch *et al.*, 2009; Muller *et al.*, 2007). The up-regulation of CXCR3 shown by pCB Tregs in combination with the absence of IFN-γ secretion, suggest a stable suppressive phenotype and correlates with the functional assays where strong inhibition of IFN-γ secretion from $CD4^{pos}$ $CD25^{neg}$ T cells was observed when they were co-cultured with pCB Tregs (Chapter 5). In accordance, the expression of CD39 in pCBTregs correlates with their suppressive function (Chapter 5) and it has also been demonstrated that $CD39^{pos}$ $Foxp3^{pos}$ Tregs are potent suppressors of Th-17 effectors (Fletcher et al., 2009). In addition, a population characterized as $CD4^{pos}$ $FoxP3^{pos}$ $ICOS^{pos}$, mediates suppression through contact with dendritic

cells in an IL-10 dependent manner (Ito *et al.*, 2008). However, although pCB Tregs upregulated ICOS expression, no IL-10 secretion was observed. Noteworthy, the study that described secretion of IL-10 from this population used ICOS-L in addition to a polyclonal stimulation. Therefore, specific co-stimulation or conditioning may be required to elicit detectable levels of IL-10 secretion, thus the capacity of IL-10 secretion from pCB Tregs cannot be excluded from this experiment.

It is known that activated T cells up regulate MHC class II expression (LaSalle et al., 1992). In agreement, pCB Tregs upregulate HLA-DR expression upon activation. It has been described that HLA-DR^{pos} Tregs represents a mature population that achieves early cell-contact suppression (Baecher-Allan et al., 2006). Additionally, the same group has shown that HLA-DR^{pos} Tregs are less likely to convert towards Th-17 effectors (Beriou et al., 2009). Moreover, different levels of Th-17 conversion from nTregs have been reported (Beriou *et al.*, 2009; Weaver *et al.*, 2009). It has been shown that the presence of IL-1 favours Tregs differentiation towards Th-17 effector T cells (Beriou et al., 2009; Deknuydt et al., 2009). Herein, is shown that CB CD4^{pos} cell subsets are more reluctant to Th-17 differentiation under the influence of IL-2 and IL-1b, compared to adult CD4^{pos} T cells. Nonetheless, due to the lower levels of IL-17 depicted among all CB CD4^{pos} cell populations and in addition to the inevitable percentage of contaminating effector T cells within the CD25^{pos} populations, a clear elucidation of the cell subset responsible for the paucity IL-17 depicted cannot be attained. However, higher cell proliferation was observed when both of these cytokines were used, independently of the source and the cell

population tested. The level of IL-17 observed from long-term expansion cultures of pCB Tregs correlates to some extent with what has been published from adult Tregs studies (Afzali et al., 2010; Beriou et al., 2009), although the levels of IL-17 shown on these studies were observed on shorter time cultures. Noteworthy, the level of IL-17 (~10%) expression among the FoxP3^{pos} population from expanded pCB Tregs, correlates with the initial 10% level of contaminating effector T cells prior expansion. Therefore, it is more likely that the contaminating effector T cells are responsible for the IL-17 detected. Regardless, expanded pCB Tregs maintain their suppressive potency.

In summary, pCB Treg, upon activation, showed a stable regulatory phenotype with beneficial properties for a proper suppressive function *in vivo*. However, long-term expansion protocols, in addition to an increase of contaminating effector T (>10% of CD4^{pos} CD25^{pos} CD127^{high}) prior expansion, cells may affect this virtue.

CHAPTER 7

pCB TREGS AMELIORATES SKIN TRANSPLANT REJECTION IN VIVO

7.1 Introduction

Currently the urge of stepping from bench to clinic, in terms of cellular immunotherapy, has elicited a scarcity of in vivo models that resemble better the clinical situation (Ishikawa *et., al*, 2005). Specifically, the preclinical models so-called ¨ humanised models ¨ allow us to explore human tissues in immune-depleted animals (Nadig *et., al*, 2010).

In order to assess the functionality of pCB Tregs in vivo, a collaboration was established with Prof. Kathryn Wood's group in Oxford. The entire process of cell preparation prior infusion and the humanized skin transplant model itself, was carried out by Issa Fadi and Joanna Wieckiewicz (members of Prof. Kathryn Wood´s group). Noteworthy, the following experiment was done at their facilities (Nuffield Department of Surgery, John Radcliffe Hospital, University of Oxford, Oxford, UK.). The CD25 isolation of five pooled CB units used in this only preliminary experiment was done at the ANRI. After the isolation process, cells were transported immediately in cRPMI in a chilled sealed compartment to Nuffield Department of Surgery research department, Oxford, UK. Once arrived, the cells were spun, placed in fresh cRPMI. Cell count and viability was performed by dye exclusion method. A total of $6.5x10^6$ cells were counted with a 95% viability. The phenotype of the resulting CD25 isolation showed similar results as described in chapter 3 with a 90% purity for $CD4^{pos}$ $CD127^{low}$ $FoxP3^{pos}$ (Figure 7.1).

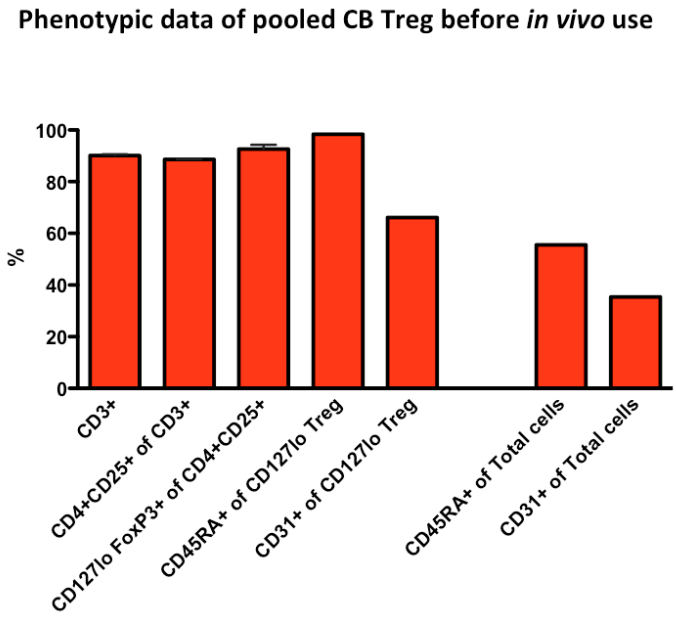


Figure 7.1 FACs characterization of the pCB Tregs prior infusion. Percentage of populations gated from lymphocyte gate using FSC and SSC.

In order to test the suppressive capacity of pCB Tregs, a previously optimized humanised mouse model of skin transplantation using healed-in human skin allografts in PBMC-reconstituted BALB.c Rag2-/- IL2rg-/- mice was used (Fadi *et., al,* 2010). A follow up of 100 days was done and graft survival was the end point of this preliminary experiment. From the total of mice used in the experiment group (n=4), one did not engrafted thus it was not included in the final analysis. Therefore, only three mice that fully engrafted from this group were included for a complete analysis. All the mice used for the positive control (n=3) showed full engraftment. As shown in figure 7.2, the positive control group rejected the skin graft as expected around 40 days post-infusion, in the other hand, the group with pCB Tregs had a median graft survival of >100 days. In addition, no GvHD was seen in the experiment group.

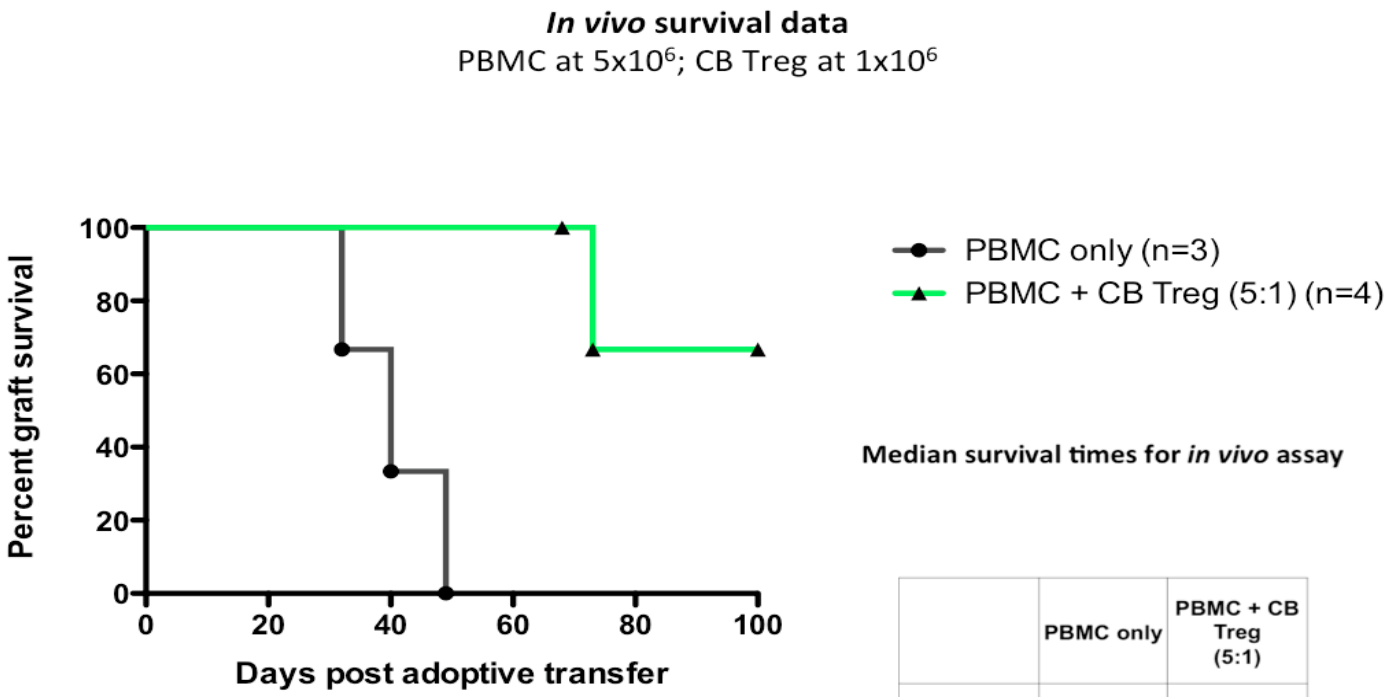


Figure 7.2 Graft survival graph (n=4). Humanised mouse model of skin transplantation using BALB.c Rag2-/- IL2rg-/- mice. A total of $5x10^6$ PBMC and $1x10^6$ pCB Tregs were infused (5:1 ratio). Control group were only infused with $5x10^6$ PBMC (n=3). Results show percentage of survival in a 100 days follow up. Median survival time is also shown between groups.

Only one mouse presented a delayed skin graft rejection on day 73. Noteworthy, most of the current data suggest that a 1:1 ratio (Effectors: Tregs) has to be obtained in order to properly ameliorate graft rejection. This preliminary experiment using a much lesser dose of adoptively transfer Tregs (1:5 ratio) in addition of the novelty of using multiple mismatch CB Tregs, showed a potent protective effect in a humanized skin transplant model. This results goes in agreement with the *in vitro* data showed in previous chapters. In summary, pCB Tregs showed beneficial effects in this pre-clinical model, further experiments are warranted in order to validate this preliminary results.

CHAPTER 8

GENERAL DISCUSSION

The evolving knowledge in transplant immunology has given the basis for the current therapeutic approaches that enables graft engraftment in HSCT and overall good 5 years graft-survival in solid organ transplantation (SOT) (Fehr *et al.*, 2004; Lechler *et al.*, 2005; Nowak, 2008). However, the morbidity and mortality due to GvHD (Socie *et al.*, 2009) and long-term immunosuppression required in SOT, in addition to the unsuccessful results so far to avoid chronic rejection (Kwun *et al.*, 2009), are still main concerns that open the field for new therapeutic options. The compelling data from preclinical models using Tregs to prevent GvHD (Edinger et al., 2003; Hippen et al., 2008), likewise, to achieve long-term graft survival in SOT (Cobbold *et al.*, 2003; Jiang *et al.*, 2006; Joffre *et al.*, 2008) without the requirement of constant immunosuppression has prompted Treg immunotherapy as a valuable option for their translation into clinical transplantation settings (Brusko *et al.*, 2008b; Hoffmann *et al.*, 2006c; Long *et al.*, 2009). This study describes the phenotype and functional properties of CB Tregs and also tested their use as a "third party" approach in transplantation settings.

Firstly, one of the main concerns is to identify the "*bona fide"* Treg from the overall Treg pool (Riley *et al.*, 2009; Seddiki *et al.*, 2006b). As mentioned, evolving studies supports that $CD4^{pos}$ $CD45RA^{pos}$ $FoxP3^{pos}$ Tregs represent the

most homogenous and stable population among the overall Treg pool (Hoffmann *et al.*, 2006b; Miyara *et al.*, 2009; Seddiki *et al.*, 2006b). However, the proportion of $CD45RA^{pos}$ Tregs decreases in an age dependent manner (Santner-Nanan *et al.*, 2008; Seddiki *et al.*, 2006b; Vukmanovic-Stejic *et al.*, 2006). In agreement, CB Tregs are mainly $CD45RA^{pos}$ (~89%) compared to adult Tregs (~18%). In addition, CB Tregs co-express CD31 (~80%), which will be considered as RTE Tregs ($CD45RA^{pos}$ $CD31^{pos}$ $FoxP3^{pos}$), which constitutively expressed a wide TCR repertoire (Junge et al., 2007; Kilpatrick et al., 2008). Recently it has been demonstrated that the natural wide TCR repertoire from Tregs is a mechanism non-redundant for the maintenance of self-tolerance (Adeegbe et al., 2010; Haas et al., 2007). Thus, the conserved wide TCR repertoire from the $CD45RA^{pos}$ $CD31^{pos}$ population in CB Tregs may be in part responsible for their better suppression shown in this study, when compared to adult third party Tregs. In addition, naïve $CD31^{pos}$ $CD4^{pos}$ T cells have shown to have longer telomeres and higher telomerase activity (Akbar *et al.*, 2007a; Kilpatrick *et al.*, 2008), hence, and in agreement with a recent study (Miyara et al., 2009), these cells have more capacity for replication and survival in vivo.

Secondly, this study shows an optimized method for CB Tregs isolation using a single step method with GMP standards that constitutively achieves high purity and yield. In addition, the inevitable level of contaminating effector T cells within Tregs isolation methods is another factor of safety-importance for the translation into clinical settings. Regardless of the great reduction in effector T cells (<9%) achieved with this method, it is evident that if a positive isolation step is done

using anti-CD25 microbeads and mostly due to the knowledge that CD25 expression is non-exclusive to Tregs, it is very unlikely to avoid a contaminating $CD25^{pos}$ $FoxP3^{neg}$ population, this in a greater extent from adult PBMCs than CBMCs. However, the phenotype of that population in CB is mainly naïve, in contrast to adult, which would express in their totality a memory phenotype. It is known that memory T cells have a lower threshold for full activation (Surh *et al.*, 2008; Takada *et al.*, 2009; Tough *et al.*, 1999). More recent studies supports that an extent of virus-specific memory T cells can cross-react to allo-antigens, creating a significant barrier for transplantation and for the use of co-stimulation blockade protocols (Bingaman *et al.*, 2004; Fairchild, 2006). Conversely, it has been shown that naïve $CD4^{pos}$ T cells are able to convert to a suppressive phenotype in the presence of TGF-β. Moreover, this study shows that in an inflammatory milieu that highly induces Th-17 conversion from adult $CD4^{pos}$ T cells, overall CB $CD4^{pos}$ T cells were highly reluctant towards this effect. Lastly, it has been reported a low incidence of severe GvHD in CB transplantation in HSCT settings, this without increasing the relapse rate (Rocha *et al.*, 2009). This effect may not only be attributed to the enriched population of Tregs within the $CD4^{pos}$ $CD25^{pos}$ fraction within CB, but also to the naïve effector T cells, which may be more easily controlled by their counterpart Treg population. Therefore, CB effector T cells in combination with a higher proportion of Tregs (9:1 Tregs: effectors) are less likely to cause an unwanted pathogenic effect once infused *in vivo*.

Thirdly, specificity has been another matter of debate in regards to Tregs immunotherapy. Noteworthy, this may be really difficult to pinpoint since

recipient T-cell allo-reactivity is a dynamic process that has the capacity to shift towards other epitopes within the allogeneic MHC molecule (epitope spreading) as well as to alloantigens expressed by the graft (Ford et al., 2009; Suciu-Foca et al., 1998). Therefore, expanding Tregs against donor APCs may not entirely produce a Treg population that will be able to suppress the variety of allo-antigens encounter *in vivo* and the process itself, may even reduce the TCR repertoire towards only the allo-antigens encounter *ex vivo* by the APCs used in culture. In accordance, it has been shown that the requirement of both direct and indirect-alloantigen specific Tregs are indispensable to prevent acute and chronic rejection (Joffre *et al.*, 2008; Tsang *et al.*, 2008), likewise in HSCT settings, polyclonal expanded Tregs are required to prevent or suppress GvHD (Hoffmann *et al.*, 2006c). Therefore, these results suggest that Treg TCR repertoire should be as wide as their counterpart allo-reactive effector T cells in able for them to properly hamper the dynamic pattern of allo-reactivity that evolves *in vivo* in the different settings. Nonetheless, alloantigen specific Tregs are also preferred in the context to avoid pan-immunosuppression within the host, which may hamper immune surveillance towards environmental pathogens and tumor cells. This statement is based on studies where chronic exposure to certain pathogens induces regulatory T cells that aid their survival within the host (Belkaid, 2008; Hasenkrug, 2003). Likewise, it has been shown that certain tumors escape immune surveillance due to the induction of regulatory T cells (Li et al., 2007; Menetrier-Caux et al., 2009; Perrone et al., 2008). However, it was demonstrated in a Treg dependent tolerance mice model, that the adoptive transfer of $CD4^{pos}$ $CD25^{pos}$ T cells did not affect the immune responses towards influenza virus within the recipient (Bushell et al., 2005). Moreover, it was shown

in a GvHD model, that the adoptive transfer of polyclonal expanded Tregs did not prevent the activation of donor allo-reactive T cells that endure effectively the Graft versus Tumor (GvT) effect, this, without compromising the beneficial effect of Tregs to abolish GvHD (Edinger et al., 2003). Thus, the risk of pan-immunosuppression occurring due to adoptively transferred Tregs is not clearly grounded at the moment, although further measures might be necessary in clinical transplant settings where a risk of tumor relapse is increased.

Additionally and in context of adoptive T cell therapy, it has been shown that a low clonal abundance within a polyclonal repertoire favors the survival and activation of naïve $CD4^{pos}$ T cells once adopted *in vivo* (Hataye et al., 2006). Hence, in support that an inverse relation between cell survival and clonal frequency occurs, it seems implausible the commitment towards protracted expansion protocols that achieves Tregs with higher TCR frequencies with the inherent inevitable outgrowth or conversion towards unwanted effector T cells that occur within these protocols. Therefore, it seems worth pursuing the modality tested in this study of *pooling* CB Tregs. Primarily because the risk of conversion or outgrowth from freshly isolated CB Tregs is lesser compared to adult Tregs, and also a low frequency repertoire would be enriched within the *pooled* CB Tregs that could resemble better and counteract the allo-reactive effector T cells.

Lastly, the evolving data in cord blood transplantation has strengthened the relevance of the cellular components within HSCT. The immature phenotype of CB cell subsets has been suggested to be responsible for that beneficial effect

shown in clinical settings (Rocha *et al.*, 2009). However and in spite of the less stringent requirement for HLA matching, a threshold of TNC or $CD34^{pos}$ cells infused, most be attain for a better outcome (Michel et al., 2003; Wagner et al., 2002). To address this matter some groups have shown promising results using the modality of double unit cord blood (DCB) transplantation in HSCT (Fernandes et al., 2007; Gutman et al., 2010; Kang et al., 2009; Verneris et al., 2009). Interestingly, it has been shown that after DCB transplantation, one CB unit fully engrafts and became dominant "single-unit dominance" (Rocha *et al.*, 2009). Regardless of the discovery of *graft versus graft* phenomena and the due elucidation involved in single-unit dominance, these results have shown that the type of cell subsets within the graft, are nonredundant for engraftment and to modulate the effector phase induced by allorecognition. Lastly and most important, these cell subsets and components allows the possibility to circumvent beyond HLA disparities in transplantation and grounds the convenience of a third or even multiple party approach in adoptive cellular therapy. In accordance, the modality of *pooling* CB Tregs tested in this study, showed that the HLA discrepancy within the CB units pooled can be intentionally used to induce allorecognition towards tolerance. Accordingly, it has been shown that matching donor and recipient on MHC II favors tolerance through the induction of Tregs (LeGuern et al., 2010). Furthermore, it has also been shown that induction of Tregs towards non-inherited HLA maternal antigens (NIMAs) takes place in early life in utero (Mold et al., 2008). These Tregs are maintained throughout life and preserved their potent suppression ability against maternal antigens. In agreement, it was demonstrated in CB transplantation an increased survival rate when donors are match to their NIMAs (van Rood et al., 2009).

Therefore, MHC II and NIMAs can initially be taken as selection criteria for optimizing CB Tregs suppression effect *in vivo.*

The study reported by Trzonkowski et al., has shown the safety of adoptively transfer of donor polyclonal expanded $CD4^{pos}CD25^{pos}CD127^{neg}$ T cells as an adjuvant therapy to treat a patient suffering of chronic GVHD (Trzonkowski *et al.*, 2009). Conversely they did not see the same effects in a patient undergoing acute GVHD (grade IV). In spite of using a currently non-GMP approved method, they showed an important decrease of FoxP3 expression from the expanded culture cells (from 90% to 40% $FoxP3^{pos}$). Lastly, they showed that multiple infusions might be required for the proper treatment of acute and chronic GVHD.

From an ongoing trial in nonmyeloablated or myeloablated recipients of two unrelated CB units transplantation, an infusion is given of ~$4x10^9$ CB Tregs from an additional partially matched CB unit (Riley et al., 2009). Results are still pending, but from a recent presentation (Regulatory T cell meeting in Beijing, China 2008) from that group, no severe GvHD was reported with apparently no complications directly associated with the Treg infusion or the HLA mismatches from the three unrelated partially matched CB units used.

In summary, findings from this study suggests that CB units fulfills the optimal properties for the isolation of *bona fide* Tregs, which can be isolated with the highest purity using a single step isolation method under GMP standards. Moreover, CB Tregs can be intentionally *pooled* and tailored under the required

matches for HLA and NIMAs for the clinical setting in hand. This strategy not only attains optimal cell numbers without the requirement of long term cultures, in addition, it allows to preserve their proliferative cellular properties and foremost, achieves a Treg pool with a wide TCR repertoire optimal for transplantation settings. Moreover, they can be either used as a first line therapy or as an adjuvant with host or donor Ag specific Tregs.

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Appendix

Source	HLA-A	HLA-B	HLA-Cw	HLA-DRB1
HC1	02/3002	51/18		1602/03
HC2	31/68	5101/5101	16/15	01/15
HC3	03/23	0702/0702	0702/0702	1501/1501
HC4	03/2901	44/44		11/07
HC5	1101/68	3501/3503	04/04	0103/1201
CB1	2601/3001	1302/3801	602/1202	1501/301
CB2	2402/2402	1801/4901	701/701	1104/1302
CB3	201/2402	4402/1402	202/501	1601/701
CB4	101/3101	5101/801	701/1501	1501/301
CB5	1101/3201	5701/4901	701/701	405/701
CB6	2402/2501	5101/801	701/1504	407/1101
CB7	201/3301	5101/4403	202/401	301/1101
CB8	301/301	5101/3501	102/401	101/1302
CB9	201/2402	5108/702	702/1602	1201/1302
CB10	205/6802	1402/4101	701/802	102/701
CB11	101/201	1517/5001	602/701	1302/806
CB12	101/3301	1402/5701	701/802	301/701
CB13	101/201	801/4402	501/701	101/1114
CB14	201/6801	5002/3508	401/602	1301/701
CB15	3002/6801	5201/5301	602/701	1102/1101
CB16	201/2402	702/4001	302/702	101/101
CB17	301/2901	5101/4501	303/602	404/701
CB18	2011/2402	3901/3901	302/702	407/901
CB19	101/201	5108/1517	701/1602	
CB20	201/3301	1401/3508	401/802	1501/701
CB21	201/201	3501/4001	304/401	301/1301
CB22	1101/3001	5101/1501	303/1502	1501/1103
CB23	301/3201	1401/1402	802/802	102/701
CB24	2301/2402	1302/4901	602/701	403/701
CB25	2301/2601	4402/3801	501/1202	
CB26	2601/6801	4403/3503	202/401	301/701
CB27	201/2402	702/4901	702/701	101/1501
CB28	201/2301	4403/3901	401/1203	1601/1104

CB29	2301/2901	4403/1503	202/1601	405/701
CB30	2402/2901	702/4901	702/701	1401/1001
CB31	201/2901	4402/4403	501/1601	101/401
CB32	301/2601	3801/3503	401/1203	1301/701
CB33	201/2901	4403/3904	1202/1601	403/1104

Appendix of section 2.8

Publications

-**Figueroa-Tentori D**, Querol S, et al. High purity and yield of natural Tregs from cord blood using a single step selection method: *J Immunol Methods* 339,228-35 (2008).

Abstract published

--**Figueroa-Tentori D**, Duggleby R.C, Querol S, Madrigal J. A. Cord Blood Tregs can be pooled and effectively suppressive in mixed lymphocyte cultures. Oral Abstract 834.. Transplantation. 2008 Jul; 86(2s): 291.

In preparation

Regulation of allorecognition by HLA disparities of cord blood CD4+ CD25+ FoxP3+ T cells

Presentations

- Regulatory T cell immunotherapy: *the more the merrier*: UCL Cancer Institute Conference, London, UK July (2009) **(Oral)**

- “Off the shelf” cord blood Tregs: practical and functional source for Regulatory T cells immunotherapy: The 2nd. Newcastle Therapeutic Tolerance Workshop, Newcastle, UK June (2009) **(Best poster Award)**

-Pooling of naïve cord blood Tregs; a new approach to achieve good qualiy and cell numbers for cellular immunotherapy: International Conference on Regulatory T cells and Clinical Application in Human Diseases, Beijing, China, October (2008) **(Poster)**

-Cord Blood Tregs can be pooled and effectively suppressive in mixed lymphocyte culture: XXII International congress of the Transplantation Society, Sydney, Australia, August (2008) **(Oral)**